GROWTH REGULATION OF OVARIAN CANCER

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This thesis is dedicated to my parents, my wife, my children and to the rest of my family who supported me by their enthusiasm and encouragement in my academic career

CONTENTS PAGE NUMBER

CHAPTER I INTRODUCTION PAGE NUMBER

LIST OF ILLUSTRATIONS

DECLARATION

I state that all the work in this thesis was performed personally unless otherwise acknowledged.

OMAR O. EL-JERBI

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LIST OF TABLES

ABBREVIATIONS

ATP	Adenosine triphosphate
Anti-PPLO	Anti-Pleuro-Pneumonia Like Organisms
BSA	Bovine Serum Albumin
CO_2	Carbon dioxide
CA-125	Carcino antigen 125
Cm2	Area in Square Centimetres
cpm	Counts per minute
Ci	Curie
^oC	Degrees Centigrade
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DHIDCCFCS	Dialysed Heat-Inactvated Dextran-Coated Charcoal Stripped Serum
DMSO	Dimethyl Sulphoxide
Kd	Dissociation Constant
DTT	Dithiothreitol
DMEM	Dulbecco's Modification of Eagle's Medium
ETN	EDTA-Tris- NaCl buffer
EGF	Epidermal Growth Factor
EGF-r	Epidermal Growth Factor-receptor
EDTA	Ethylene diamine tetra- acetic acid
FIGO	Federation International Gynecologic and Obstetrics
FCS	Foetal Calf Serum
fmol	Femtomoles

FSH	Follicle Stimulating Hormone
g	Gravitational force
HCl	Hydrochloric acid
HEPES	Hydroxyethyl-piperazine ethane sulphonic acid
125_I	Iodinated Label
KDa	KiloDaltons
l	Litre
LH	Luteinizing Hormone
µg	Microgram
µl	Microlitre
µM	Micromolar
mCi	Millicurie
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
m mol	Millimoles
M	Molar
mol. wt	Molecular Weight
m RNA	Messenger Ribonucleic acid
OD	Optical Density
E2	Oestradiol
OCCSA	Ovarian Cancer Cell Surface antigen
PBS	Phosphate Buffered Saline
PMSG	Pregnant Mares Serum Gondotrophin
RIA	Radio-Immuno-Assay

NaCL	Sodium Chloride
SDS	Sodium Dodecyl Sulphate
TGF-α	Transforming Growth Factor alpha
TGF-β	Transforming Growth Factor beta
TGF-βr	Transforming Growth Factor beta receptor
UV	Ultraviolet (light)
V	Volume
V/V	Volume per volume
W/V	Weight per Volume
WHO	World Health Organisation

SUMMARY

Ovarian cancer arises almost exclusively in epithelial cells derived from the surface of the ovary. It is thought that the continual damage caused by ovulation (break of the epithelial surface layer to allow release of the follicle) and the subsequent repairs are the prime factors in promoting ovarian epithelial cancer. The prime promoter of growth repair is epidermal growth factor (EGF). EGF works through the EGF receptor (EGFr) and EGFr has been detected in about 45% of ovarian cancer.

Epidermal growth factor and transforming growth factor alpha are two peptides which bind to the epidermal growth factor receptor. Although the literature shows that epidermal growth factor (EGF) was detected in less than 30% of ovarian cancers, transforming growth factor alpha (TGFα) was present in 88.5%. To investigate the possible role of growth factors in ovarian carcinoma, the OAW42 epithelial ovarian cancer cell line (previously established from ascites of patient with ovarian cancer) was used as a model. To allow these studies to be extended to the effects of drug resistance, PEO1 and PEO1cddp cells were used (PEO1 was a platinum sensitive cell line, PEO1cddp a platinum resistant cell line).

Growth stimulation of OAW42 cells over 48 and 72 hours by TGFα reached a maximum of 42 and 19.6% above control respectively at 10 ng/ml dose.

Growth stimulation by TGFα was seen in PEO1 cells as early as 24 hours, reaching a maximum of 35% above control at 25 ng/ml.

Growth response to EGF showed that OAW42 cells responded positively to the influence of EGF after 48 and 72 hours reaching a maximum of 34% above control at 10 ng/ml. PEO1 cells again showed earlier stimulation of growth by EGF reaching a maximum of 18.9% after 24 hours, 30.8% at 48 hours, and 16.1% after 72 hours at dose of EGF (2.5 ng/ml). Both cell lines were, EGFr positive, as expected. After 24 hours exposure of PEO1cddp cells to EGF, a slight stimulation of growth was seen at a dose of 5 ng/ml (about 6.3% above control). After 48 hours no stimulation of cell proliferation seen. At 72 hours exposure PEO1cddp cell growth was inhibited by all concentrations of EGF, suggesting that acquision of drug resistance was also associated with changed response to EGF.

TGFβ, a known inhibitor of the proliferation of normal ovarian epithelial cells was shown to inhibit growth of OAW42 cells over 96 hours reaching a maximum of 35% at 5 ng/ml. After treating OAW42 cells with luteinzing hormone (LH), cells showed inhibition of growth reaching a maximum of 21, 26.2, and 30.1% after 24, 48, and 72 hours respectively at 60 iu/l dose of LH. PEO1 cells showed growth inhibition, reaching a maximum of 34.2, 26.9, and 32.8% below control after 24, 48, and 72 hours respectively at 60 iu/l dose of LH. Anti-TGFβ polyclonal antibody (5 μl/ml) reversed LH-induced inhibition of growth. A further increase of anti-TGFβ antibody up to 30 μl/ml gave growth stimulation of 11.3% above control cells suggesting that TGFβ is normally having some inhibitory effects on the cells under control conditions in addition to mediating the LH response.

Cancer and other hyperproliferative diseases often show elevated protein tyrosine kinase (PTK) activity, related to the increased activity of growth factor receptors. In the present study, there was growth inhibition of OAW42 cells over 72 hours by blocking PTK with an EGF receptor specific tyrosine kinase inhibitor, reaching a maximum of 38.3% below control cell growth at 3.0 μM dose. PEO1 and PEO1cddp cells were also growth inhibited by increasing concentrations of TKI from (0.5 to 3.0 μM) reaching a maximum of 55.0 and 59.3% below control respectively at 3.0 μM dose. This confirmed the importance of EGF as a growth promoter in at least some ovarian cancers.

Electron microscopy was used to study the hormonaly-induced changes in the cellular morphology of ovarian cancer cells. The actions of LH were compared with those of epidermal growth factor (EGF). Exposure of OAW42 cells to EGF at 10 ng/ml dose for up to 72 hours showed swollen mitochondria, more evidence of polysomes and more stubby microvilli, in keeping with the growth response to EGF seen after 72 hours incubation. The effect of LH showed no evidence of swollen mitochondria, no prominent polysomes and few stubby microvilli, this time reflecting the growth inhibition of higher doses of LH.

On the basis of the results described in this thesis, new therapies for ovarian cancer may be based on either regulation of the EGF receptor or promotion of the action of TGFβ.

CHAPTER I

INTRODUCTION

1.1 An Introduction to Cancer

About 140 years ago a German microscopist, Johannes Mueller, showed that cancers were made up of cells, a discovery which began the search for changes which would help to pinpoint the specific differences between normal and cancer cells. The visible tumour is the end result of a whole series of changes and may have taken many years to develop. Cancer is not a single disease but many diseases sharing a single name. It is the spread of malignant tumours, particularly to vital organs, that makes them both difficult to treat and potentially lethal.

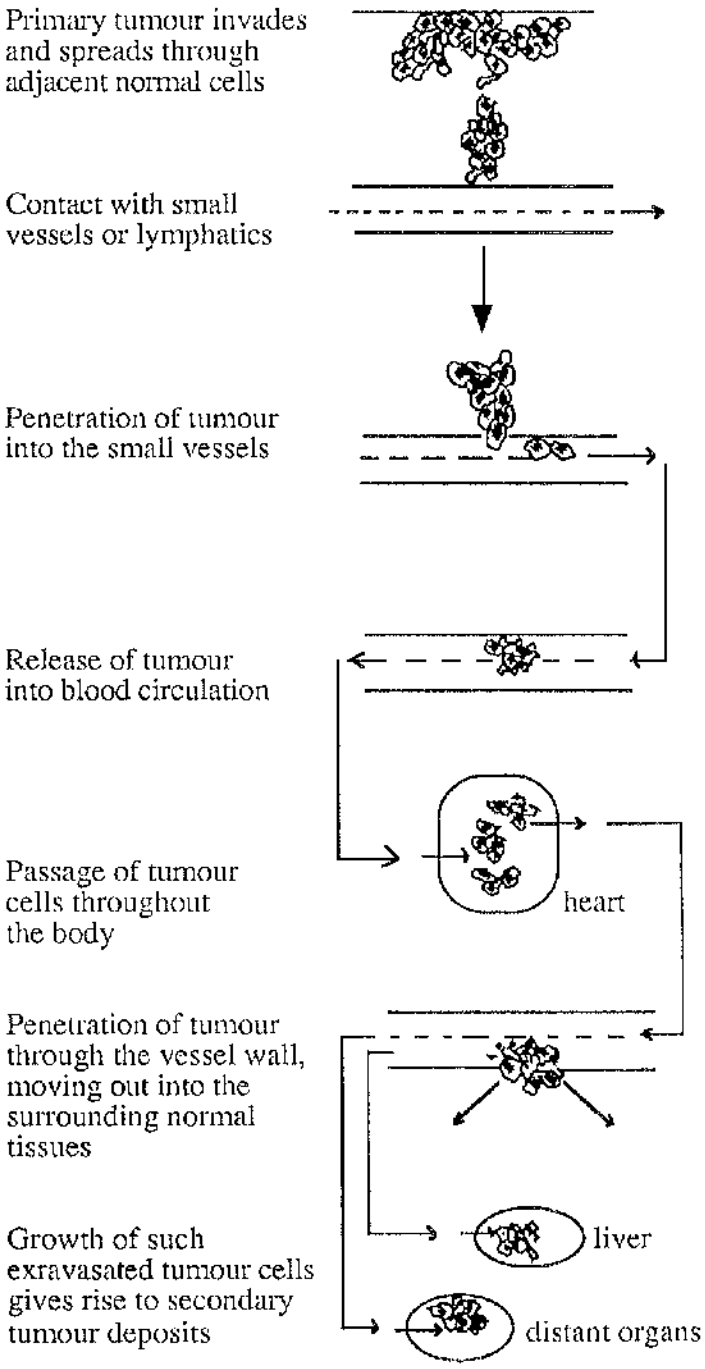
Tumours can be either malignant or benign. Those that remain localised can usually be cured by surgery or radiotherapy e.g patients with large basal cell skin cancer can be treated successfully as these tumours rarely invade deep into the skin or spread to lymph nodes.

Some tumours, especially deep-seated ones, can become quite large (up to 1-2 cm diameter) with no symptoms being noticed during a routine medical examination. Malignant tumours escape normal biological controls and, further, support themselves on nutrients which were intended for healthy cells or organs. Malignant tumours will initially invade and destroy the surrounding tissue, then enter both the lymphatic supply and the blood stream.

1.2 Spread of Cancer

Spread of cancer cells (metastatic cells) may occur via the blood stream or the lymphatic channels or across body cavities such as the pleural and peritoneal spaces setting up secondary tumours (see figure 1), causing different effects in different part of the body e.g a lung tumour may invade the chest wall causing pain on breathing due to pleural irritation. Bone metastasis is very common in breast cancer but very rare in cancer of the ovary, which presumably reflects the fact that metastatic cells are seeking a growth environment similar to that present in the parent tissue.

Figure 1. The spread of malignant tumours.



1.3 Some Factors Related to Carcinogenesis

Many different factors are involved in the development of tumours: a cancer producing agent (carcinogen) and presumably promoting agents must be present, though not necessarily at the same time. These factors may be chemical or physical. For example, ultraviolet light can cause skin cancer in caucasians exposed to tropical sun light. Identified chemical carcinogens include hydrocarbon carcinogens present in coal and tar and a series of chemicals used in the rubber industry, such as 2 naphthylamine, benzidine and 3-dichloro benzidine. Cigarette smoke remains the most common source of carcinogen. Alcohol consumption can cause cancer of the oesophagus and larynx.

Animal experiments suggest that viruses may be associated with the initiation of some cancers e.g the leukaemic virus group. Viruses also seem to be associated with some types of human cancer e.g. cervical cancer (HPV 16 and 18). Some tumours (e.g. a rare malignant tumour of the retina occurring in infant-retinoblastoma) are inherited through gene deletion in the parental DNA. Other cancer families inherit mutations in certain genes e.g. the BRCA-1 tumour suppressor gene on chromosome 17q was identified and shown to be responsible for some cases of hereditary breast/ovarian cancer families (Berchuck *et al* 1995). It has been estimated that women who carry mutations to the BRCA1 gene on chromosome 17q have 60% lifetime risk of ovarian cancer. A series families with either breast or ovarian cancer, identified 40% with BRCA1 mutations (Simard *et al* 1994).

1.4 Introduction to the Ovary and to Ovarian Cancer

There are two ovaries situated in the lower abdomen, one on each side of the uterus. Each ovary contains numerous follicles within which the ova develop (see figure 2). Normally, there is only one mature follicle in the ovary prior to ovulation. The ripening follicle contains a large fluid cavity that distends the surface of the ovary.

Figure 2. The mature mammalian ovary

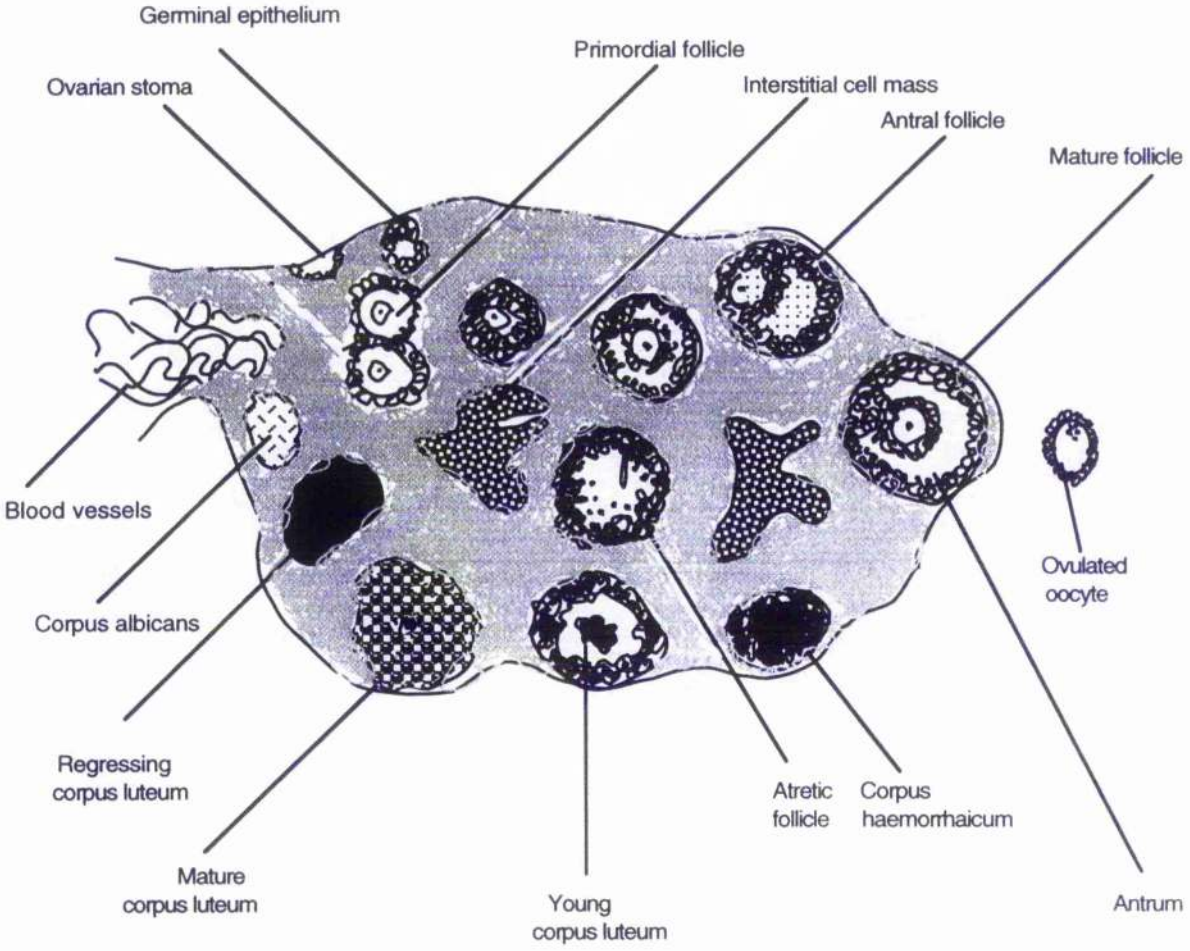


Diagram of a mammalian ovary, showing the sequential development of a follicle, formation of a corpus luteum, and in the centre,follicular atresia. A section of the wall of a mature follicular is enlarged at the upper right. The interstitial cell mass is not prominent in primates. [Adapted from Katzung BG Basic and Clinical Pharmacology, 1987].

The follicle secretes oestrogen and small amounts of androgen. After ovulation a corpus luteum forms at the site of the ruptured follicle and secretes progesterone. Oestrogen and progesterone regulate the changes in the uterus throughout the menstrual cycle and pregnancy. A repair mechanism stimulates growth of the surface epithelium in the area of the damage caused by ovulation.

1.4.1 Functions of The Ovary

The ovary has two main functions in the body, (a) the gametogenic function of production of the female germinal cells, (b) an endocrine function, consisting of the elaboration of female hormones (oestrogens, progesterone and relaxin) and some androgens. Both functions are performed, to a large extent, by the same ovarian cells. The maturation of germinal cells occurs in the evolving follicles. The elaboration of oestrogens is accomplished by the theca interna layer, with the final step (aromatisation) taking place in the granulosa cells of evoluting and involuting follicles and by the theca lutein cells of corpora lutea. Progesterone is produced by the granulosa lutein cells of the corpora lutea, which also produce relaxin ; the hilus cells and theca cells produce androgens.

1.4.2 The Effect of LH and FSH on the Normal Ovary

The processes of differentiation, function and degeneration in the cells of the ovary appear to be under the influence of secretion of the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Findlay and Risbridger 1987).

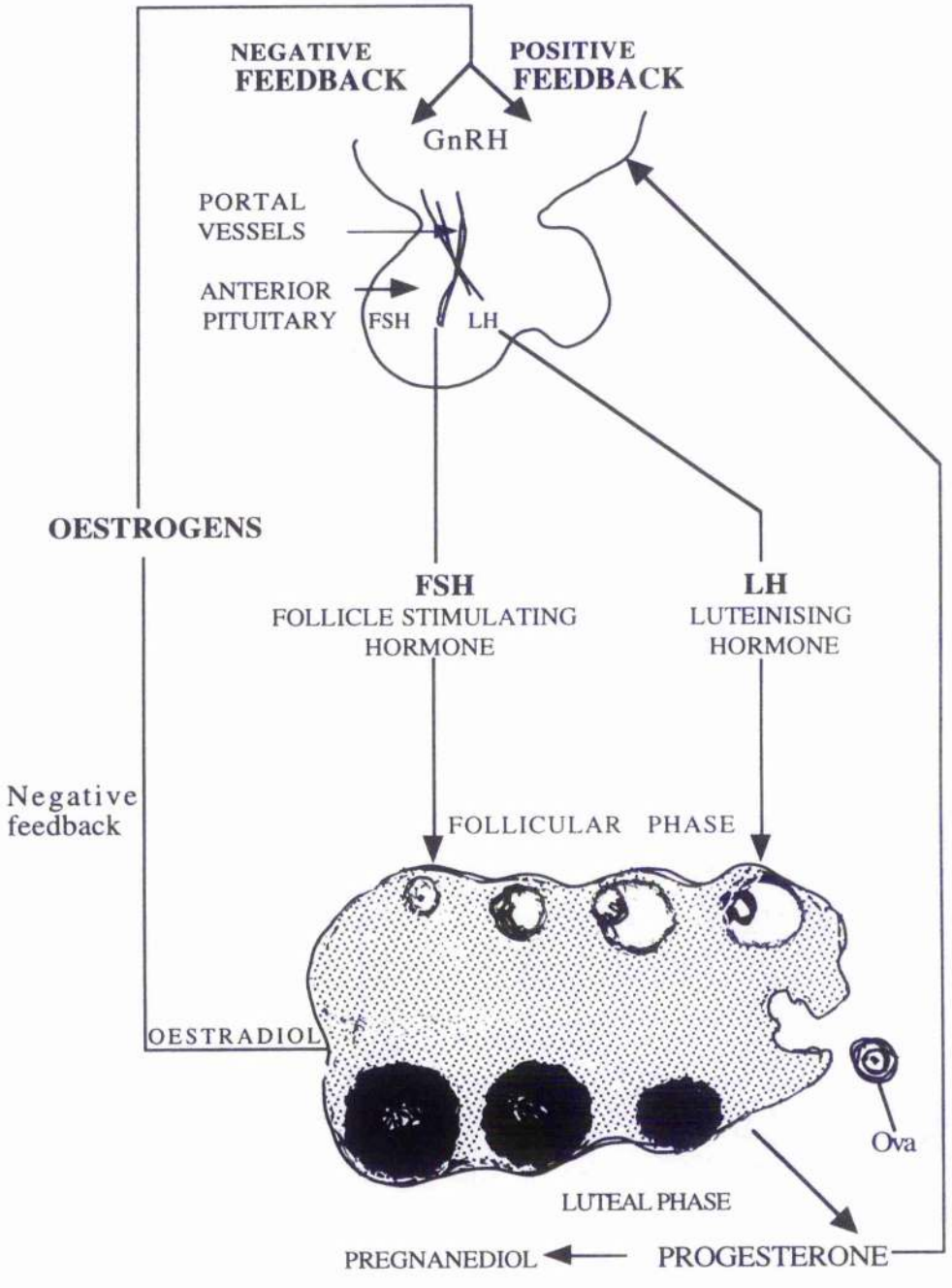
The ovary and testis share homology of structure and function in the production of gametes and in the regulation of steroidogenesis. Normal function of both the ovary and the testis is dependent on the pituitary-synthesized gonadotrophins (Ronin *et al* 1989). These are glycoprotein dimers, which share a common α-subunit. Follicular maturation is also under the control of these gonadotrophins.

These glycoproteins act through their receptors on their target cells, indicating the presence of functional gonadotrophin receptors at an early stage of follicular development (Asem *et al* 1992). FSH also regulates granulosa cell function during the follicular phase of development (Cahill *et al* 1985; Carson *et al* 1988).
The LH surge that promotes ovulation involves local release of prostaglandins, together with FSH and LH induced release of plasminogen which mediates the release of the oocyte from the follicle wall (Beers *et al* 1985).

1.4.3 Normal Pituitary and Ovarian Relationships

During the normal ovarian cycle, oestrogen secretion down-regulates FSH and LH. After the menopause feedback inhibition on pituitary peptide hormone synthesis is lost such that the pituitary produces increased amounts of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (5-15-fold above normal pre-menopausal levels) for a number of years after the climacteric (Monroe *et al* 1977). This suggests that FSH and LH may contribute to the development of ovarian cancer as the majority of ovarian cancer patients are post-menopause. Nevertheless, the marked changes in relative risk of ovarian cancer in relation to age at menopause might suggest that the initial events in ovarian carcinogenesis occur before the menopause. The ovary secretes a variety of steroid hormones, though only oestradiol-17β and progesterone are known to have established feedback roles in controlling the secretion of pituitary gonadotrophins, see (figure 3). Oestradiol-17β, which is formed within the ovarian follicle, has been shown to have direct actions on follicular cells such as influencing the activities of various steroidogenic enzymes (Findlay and Risbridger 1987). In both phases of the ovarian cycle it is the principal steroid inhibiting the release of gonadotrophins (LH and FSH) from the anterior pituitary (negative feedback). Oestradiol in the female will also provoke a discharge or surge of LH (positive feedback) at mid-cycle (Diersehke *et al* 1974).

Figure 3. Hormonal control of ovarian function



The pituitary gland produces gonadotrophin hormones LH and FSH which act to stimulate the ovary to produce oestrogen and progestrone, which then act on target tissues to induse a response. In addition, oestrogen is capable of controlling release the gonadotrophin hormones by feed back inhibition.

A failure of positive feedback to the hypothalamus at mid-cycle will result in a failure of ovulation and accounts for the occurrence of anovulatory cycles in the few months after the menarche in rhesus monkeys and in adolescent girls (Dierschke *et al* 1974).

Progesterone has little effect on the release of gonadotrophins but inhibits the ability of oestrogen to provoke positive feedback (Dierschke *et al* 1973).

Ovarian androgens (mainly androstenedione) may influence the release of gonadotrophins, either directly, or by extra-glandular aromatization to oestrogens (Martenz *et al* 1975).

1.5 Epidemiology of Ovarian Cancer in the West and in Libya

The highest ovarian cancer rates are reported in highly industrialised countries, except Japan where rates of death from ovarian cancer are the lowest in the world (Bull *et al* 1965,1968). Japanese migrants to USA and their first-generation offspring in the United States have a higher incidence of ovarian cancer than Japanese women in Japan, although the incidence is still lower than that in the white women of the United States. Ovarian cancer rarely occurs before menarche and is unusual before age 20, but the rate of occurrence tends to increase significantly thereafter. In the age range 40-44, the incidence is 15.7 per 100,000 women, while the incidence increases to 54 per 100,000 women in the 75 to 79 age group. Disordered endocrine function may contribute to the development of ovarian cancer. A higher incidence of epithelial tumours has been noted in women with a lower mean number of pregnancies, in nulliparous women, and in women with a history of infertility (Jely *et al* 1974). Repeated trauma to the surface of the epithelium during normal ovulation increases ovarian cancer risk (Fathalla 1971). High circulating levels of pituitary gonadotrophins are thought to be active in ovarian cancer development and the reduction in plasma gonadotrophin levels in oral contraceptive users is assumed to be responsible for the decrease in the risk of ovarian cancer in pill users though reduction in the numbers of subsequent wound repairs is a more likely explanation (Cramer *et al* 1983).

An increasing risk of ovarian cancer is associated with nulliparity, non-use of oral contraceptive, and increasing ovulatory years (Whittemore 1993).

The relative risk of ovarian cancer is 1.0 for women who reach menopause before age 45, after which it rises rapidly to 4.5 for women whose menopause is 55 years or later suggesting some increase in carcinogen secretion from older ovaries.

The risk of ovarian cancer decreases with each additional pregnancy (Kvale *et al* 1988) and (Hartge *et al* 1989). Most studies have noted a decreased risk among women who breast feed (Hartge *et al* 1989) and (Gwinn *et al* 1990). Oral contraceptive use, particularly for 5 years or more, has also been consistently found to result in reduction in risk (Hankinson *et al* 1992). There is a small decrease in ovarian cancer risk with even 1year of oral contraceptive use, but longer durations of use are needed for a substantial risk reduction (Susan *et al* 1992). Both nulliparous and parous women who use oral contraceptive have a decreased risk of ovarian cancer. The incidence of epithelial ovarian cancer is reduced by approximately 30% in oral contraceptives users. However the risk of ovarian cancer is associated with a 50% reduction in long-term users over five years of use (Vessey and Painter, 1995) and 70% in those taking oral contraceptive for more than 97 months.

Unprotected intercourse and/or prior use of infertility medication increased risk of invasive ovarian cancer, although age at first live birth, age at menarche, age at menopause, and oestrogen replacement use were not strongly associated with risk, while pregnancy, breast feeding, and oral contraceptive use are associated with risk reduction. An underlying hormonal imbalance might be responsible for infertility and, rather than ovulatory activity, may be responsible for the increased incidence of ovarian cancer observed in nulliparous women (Whittemore *et al* 1989). Women who have had one to two pregnancies have a risk of 0.49 to 0.97 and women with three or more pregnancies have a relative risk of 0.35 to 0.76. Each additional pregnancy appears to lower the risk by about 10% (Greene *et al* 1984).

Cancer of the ovary and breast appear to share some common etiologic factors.

Women with breast cancer have twice the expected risk for ovarian carcinoma, women with ovarian cancer have a three-fold to four-fold increase in the incidence of breast cancer (Fraumeni *et al* 1974).

Epithelial ovarian cancer accounts for about 90 percent of all malignant ovarian tumours (Berek *et al* 1989). This form of ovarian cancer often spreads without symptoms, and in three quarters of patients the disease is not diagnosed until it has spread beyond the pelvis. Approximately 70% of patients present with advanced stage disease at diagnosis and 85% of them eventually die as a result of their disease (Richardson *et al* 1985).

Ovarian cancer is the fourth commonest cause of cancer death in women and the leading cause of gynecologic cancer death in the United States (Silverberg 1983). More U.S. women die from ovarian cancer each year than from cervical and endometrial carcinoma combined (Boring *et al* 1992). U.S. incidence and mortality estimates for 1992 indicate that 21,000 new patients are diagnosed yearly and 13,000 women die from this disease. In the UK there are 4,000 new cases diagnosed every year. Overall five-year survival rate is 28% and there are 3,000 deaths each year. In contrast to western women, malignant tumour of cervix is the commonest malignant tumour of the genital tract of the Libyan women (Table 1).

The reasons for higher frequency of carcinoma of cervix in Libyan women may be attributed to early marriage, multiparity and lack of screening. The most common ovarian neoplasms in Libyan women were the germ cell tumours (51.1 and 47.7% in Tripoli and Benghazi respectively (Table 2) (Faizuddin *et al* 1989) in sharp contrast to that in Western women in whom germ cell tumours represent only 2% to 3% of total ovarian cancers (Woodruff *et al* 1968).

Table I. Prevalence of tumours of female genital tract in Tripoli and Benghazi

Tripoli			
Site of tumour	Number	Benign	Malignant
Ovary	176 (37.9%)	143 (81.7%)	33 (18.7%)
Uterus	213 (45.9%)	186 (87.3%)	27 (12.3%)
Cervix	66 (14.8%)	2 (3.1%)	64 (96.9%)
Vulva	9 (1.94%)	0	9 (100%)
Total	464	331 (71.3%)	133 (28.66%)

Benghazi			
Site of tumour	Number	Benign	Malignant
Ovary	132 (24.4%)	93 (70.5%)	39 (29.5%)
Uterus	320 (59.2%)	272 (85%)	48 (15%)
Cervix	70 (12.9%)	24 (34.3%)	46 (65.7%)
Vulva	19 (3.5%)	0	19 (100%)
Total	541	389 (71.9%)	152 (28.1%)

Table II. Epithelial and Germ cell tumours in Tripoli and Benghazi.

	Tripoli	Benghazi
Epithelial tumours	62 (35.3%)	48 (36.36%)
1. Serous and mucinous	55 (31.25%)	46 (34.84%)
Benign (adenoma)	46 (26.14%)	34 (25.75%)
Malignant (carcinoma)	9 (5.%0	12 (9.08%)
2. Brenner	2 (1.14%)	-
3. Undifferentiated	3 (1.7%)	-
4. Cystadenofibroma	2 (1.14%)	2 (1.15%)
Germ cell tumours	90 (51.14%)	63 (47.7%)
1. Benign cystic teratoma	82 (46.6%)	52(39.39%)
2. Malignant teratoma	2 (1.14%)	4 (3%)
3. Cystic teratoma with malignant change	-	4 (3%)
4. Dysgerminoma	6 (3.4%)	3 (2.27%)
Total number of tumours	176	132

1.6 The Biology of Ovarian Cancer

At the time of ovulation, ovarian surface epithelial cells produce large lysosomal bodies that migrate to the basal region of the cells. These large bodies are excreted from the basal surfaces of the cells before ovulation. It is believed the lysosomal bodies contain enzymes that participate in the breakdown of the tunica albuginea and thus aid in follicular rupture (Bjersing *et al* 1975).

The process of ovulation results in a wound at the ovarian surface that is repaired by rapid growth of ovarian surface epithelial cells (Nicosia *et al* 1991). This repair process is thought to be stimulated by epidermal growth factor (EGF).

There is also potential for various hormones and other growth factors to regulate growth and function of ovarian surface epithelial cells, as suggested by the presence of appropriate receptors for many of these regulatory substances in both the normal and malignant ovarian surface epithelial cells (Berchuck *et al* 1991) and (Bauknecht *et al* 1988).

1.6.1 Hereditary Ovarian Cancer

The present limited data suggested that the life-time risk to a sister or mother of a patient in a family, where two or more close relatives are affected, is about 40% and that roughly half this risk occurs before the age of 50. This is explained on the basis that there is a 50% chance of inheriting the gene and 80% chance that a gene carrier will develop ovarian cancer in her lifetime. The risk of developing ovarian cancer with one first-degree and one second-degree relative or two second degree relatives is less than 50% (Piver *et al* 1990; 1991).

The biology of familial ovarian cancer is very poorly understood. Between 5% and 10% of all ovarian carcinomas are hereditary (Lynch *et al* 1987) and (Schild, Kraut and Thompson 1988). Some hereditary ovarian cancer involves only segregation of ovarian cancer but others show additional forms of cancer, such as carcinoma of the breast in the breast/ovarian cancer syndrome.

Women with a family history of breast, uterine or ovarian cancer in a mother or sister showed a highly significant increased risk of ovarian cancer (Mori *et al* 1988). The association of ovarian cancer with breast cancer in families suggests that genetic variation in the metabolism of the steroid hormones could be an important factor in giving rise to inherited susceptibility to both cancers. Relative risks of 3.6 for first-degree relatives and 2.9 for second-degree relatives were reported (Koch *et al* 1989).

There are three types of hereditary ovarian cancers: site specific ovarian cancer, breast-ovarian cancer syndromes and Lynch II syndrome, which is a combination of lynch I hereditary colon cancer and the frequent occurrence of ovarian, breast and uterine cancer (Lynch *et al* 1985 and Lynch *et al* 1986). Early detection of the risk of ovarian cancer will depend on finding the abnormal gene (Narod *et al* 1991). Narod and colleagues investigated five large families with a predisposition to cancer of the breast and ovary. BRCA1 gene has focused attention as one of the genes responsible for some cases of inherited breast and ovarian cancer (Miki *et al* 1994). It is estimated that 80% of families with the breast-ovarian syndrome were due to the BRCA1 locus (Feunteun *et al* 1993, Easton *et al* 1993).

1.6.2 Early Detection of Ovarian Cancer

Ovarian cancer is a disease that remains without a reliable screening technique for early diagnosis. Early detection of epithelial ovarian cancer generally still relies on the finding of a pelvic mass on physical examination (Jacobs *et al* 1990). Screening techniques using ultrasonography and the serum tumour marker CA 125, although widely available, have not greatly enhanced our ability to discover such cancers before their dissemination. CA 125 is a 200-kD glycoprotein found as a surface protein on ovarian cancer cells (Davis *et al* 1986) and (Bast *et al* 1983). The monoclonal antibody used in the assay was raised against the antigen OVCA 433 prepared from a cell line of a papillary serious cystadenocarcinoma of the ovary. CA 125 has been used as a marker to monitor the response of ovarian cancer to treatment as well as to distinguish malignant from benign pelvic masses (Bast *et al* 1983) and (Jacobs *et al* 1989).

The use of serum levels of CA 125 combined with vaginal and or abdominal sonography, as a screening test for ovarian cancer, remains controversial because of a significant number of false positives. In general, monoclonal antibody assays developed so far lack sufficient sensitivity and specificity for reliable early diagnosis of ovarian cancer (Runowicz *et al* 1992).

1.7 Treatment of Ovarian Cancer

Early ovarian cancer is almost always clinically silent and so normally presents at an advanced stage with intraperitoneal metastasis. Treatment of advanced stage disease has been almost wholly ineffective due to the frequent development of resistance to available chemotherapeutic regimens. Taxol is currently registered for use in ovarian cancer after failure of previous chemotherapy including platinum compounds (McGuire, *et al* 1989, Sarosy, *et al* 1992).

1.7.1 Surgery

Surgery will continue to play an integral role in the management of malignancy including ovarian cancer. It is plays an important role in both the staging procedure and in the removal of as much tumour as possible (Alders *et al* 1993). It can be helpful in selecting patients for more extended treatment and saving others from unnecessary over treatment. Currently, the extent of residual disease, after surgery is completed, is the best available index of prognosis (greater than 2cm residual disease = poor prognosis).

1.7.2 Prophylactic Oophorectomy

Prophylactic oophorectomy has some times been recommended to women with a strong family history of ovarian cancer and others have suggested performing such an operation in women over 45 years who are undergoing a hysterectomy for some other reason. Lewis and Davison (1969) described a family of five sisters and their mother, all with ovarian carcinoma. Prophylactic oophorectomy was performed on five women from the next generation. None of these individuals showed histologic findings consistent with ovarian carcinoma.

The risk of ovarian cancer in women from families with hereditary ovarian cancer syndromes is sufficiently high to warrant prophylactic oophorectomy (Kerlikowske *et al* 1992).

The ovary is the major source of oestrogen in women, (see figure 4). Oestrogen receptors have been detected in both normal and malignant ovarian surface epithelial cells (Nicosia, *et al* 1991) and (Hamilton *et al* 1992). The complexity of ovarian surface epithelial cell regulation is indicated by the finding of oestrogen production by ovarian cancer cell lines (Wimalasena, *et al* 1982, Poels, *et al* 1989).
Oestrogen replacement therapy has not been associated with increased risk for ovarian cancer (Hoover, *et al* 1977, Adam, *et al* 1989).
Presence of functional oestrogen receptors is associated with improved prognosis in ovarian cancer patients (Harding *et al* 1990)
After oophorectomy, women will generally require oestrogen replacement therapy. Oestrogen therapy is associated with a decreased risk for fractures (Naessen *et al* 1990) and a decreased risk for coronary heart disease (Stampfer *et al* 1991). However, it is important to consider how this oestrogen replacement therapy might influence risk of breast cancer.

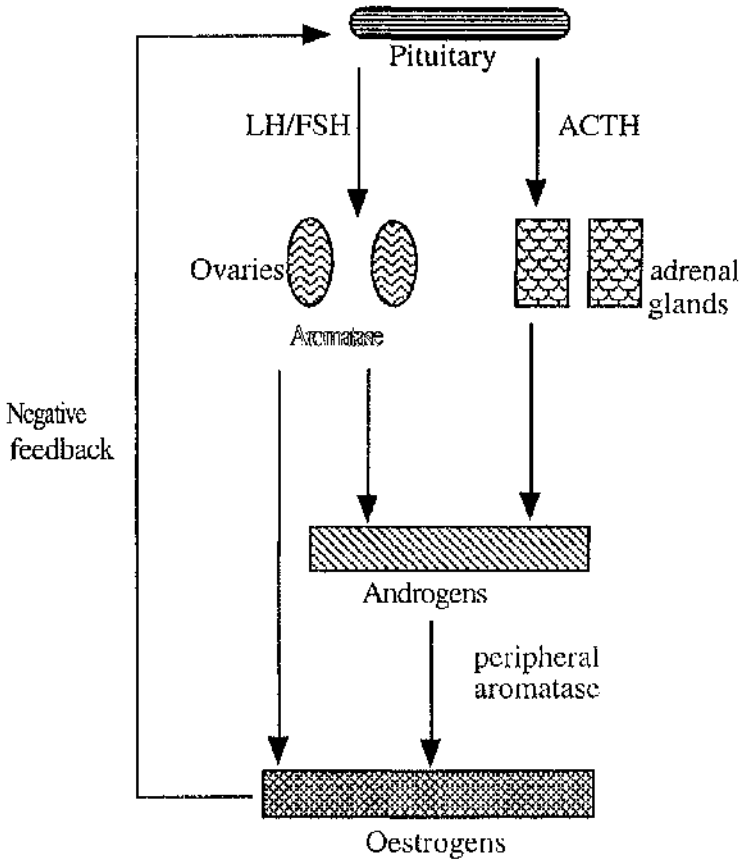
1.7.3 Tubal Ligation and Hysterectomy

Tubal ligation and hysterectomy were associated with reduced risk (Alice *et al* 1992). Tubal ligation and hysterectomy may protect against ovarian cancer by both impairing ovarian function and by preventing ovarian exposure to exogenous carcinogenic agents (such as asbestos talc) that enter the peritoneal cavity through the vagina (Whittemone *et al* 1992).

1.7.4 Chemotherapy

The treatment of patients with ovarian cancer has previously varied enormously depending on the clinician's knowledge and use of various chemotherapeutic drugs, either as single agents or multiple agents and his relationship with mainly a medical oncologist and less frequently a radiotherapist. The most effective drugs are Cis-platinum and carboplatin used, either alone, or in combination with other drugs.

Figure 4. Oestrogen synthesis



Gonadotrophins: Luteinizing hormone (LH), Follicale stimulating hormone (FSH) are bind to receptors in the ovary. LH stimulates oestrogen and progestrone prodution from the ovary. Development of the ovarian follicle is largely under FSH control, and the secretion of oestrogen from this follicle is dependent on both FSH and LH.

Cisplatin and carboplatin are the mainstays of therapy in the management of advanced-stage epithelial ovarian carcinoma (Muggia *et al* 1989 and Reed *et al* 1990). Six pulses of chemotherapy are usually given one month apart and response is observed by monitoring CA-125 levels and through pelvic/abdominal assessment. Chemotherapy for advanced ovarian cancer with combinations of drugs that contain cisplatin appears to yield a substantial proportion of long-term survivors (Neijt *et al* 1987). Use of Taxol has recently given good responses in platinum-resistant patient. Over the past 15-20 years, the overall mortality for ovarian cancer has improved, particularly for younger patients (Hole *et al* 1991).

1.8 Growth Factors and Ovarian Cancer

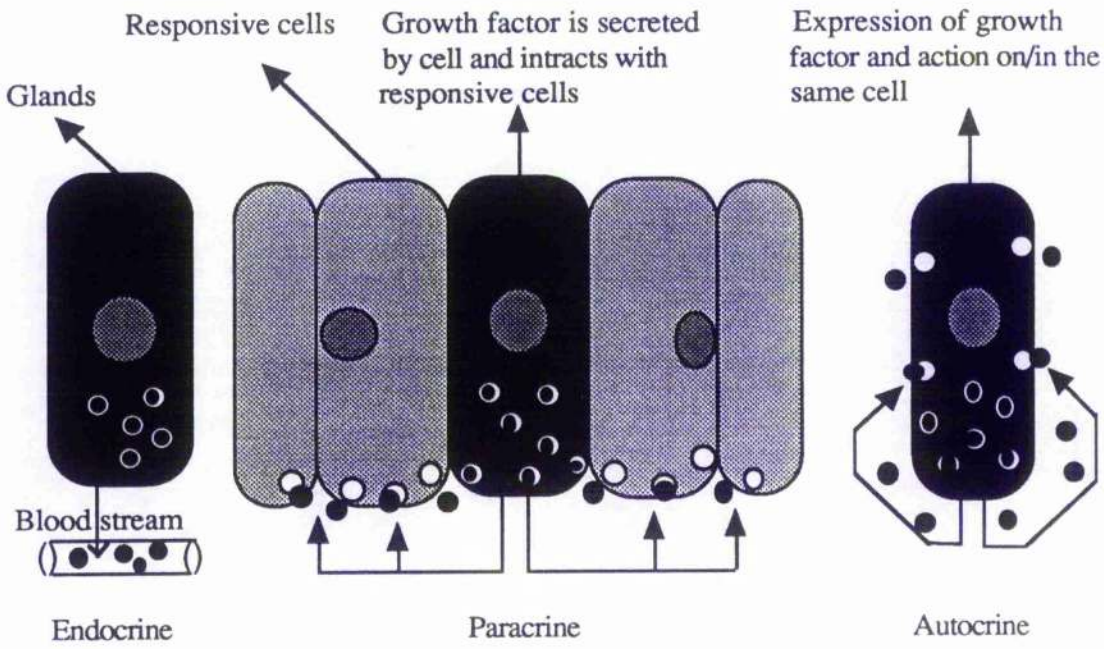
1.8.1 Growth Factors Activities in normal and malignant epithelia.

The action of hormones is mediated, at least in part, by the regulation of local growth control mechanisms. These may be autocrine and/or paracrine in their mode of action see (figure 5). Experimental evidence suggests that growth factors play extensive roles in the growth of ovarian epithelial cells following the injuries caused by ovulation. If there is a transformed cell in the area of the injury repair, then may be the onset of ovarian cancer promotion.

1.8.2 Epidermal Growth Factor

Epidermal growth factor (EGF) is a 53 amino acid polypeptide with molecular weight of approximately 6,000. TGF-α shows significant homology with EGF (Burgess 1989). Both appear to exert their biological effects by binding to high affinity EGF-receptors. EGF and/or TGF-α are present within most ovarian tumours (Bauknecht *et al* 1989 ; Leake *et al* 1990).

Figure 5 . Synthesis and delivery of growth factors



In post pubertal sheep, a depilatory dose of EGF (4.2μg/kg/hour) for 48 hours decreased LH and oestradiol (Show *et al* 1985). A similar EGF infusion inhibited ovulation and lowered oestrogen concentrations in pregnant mares serum gondotrophin (PMSG)-treated ewes. Subsequent non stimulated cycles were apparently normal (Redford *et al* 1987). These observation confirm that EGF action is not simply a local stimulation of mitosis.

EGF has been shown to be a potent mitogen for a variety of cells including rodent, porcine and human granulosa cells (Takeshi *et al* 1993).

In addition to its mitogenic property, it is evident that EGF regulates a variety of cellular functions. There is simultaneous expression of EGF and EGF receptor, not only in the granulosa and thecal cells, but also in the stromal cells surrounding the corpus albicans. It is highly possible that an autocrine control system wherein EGF serves as the signal may exist throughout the life of the ovarian follicle.

EGF has been shown to stimulate the *in vitro* growth of malignant ovarian tumours specimens (Singletary *et al* 1987). Normal human ovarian epithelial cells express high-affinity epidermal growth factor receptors and epidermal growth factor consistently acts to stimulate DNA synthesis in these cells (Rodriguez *et al* 1991). Ovarian epithelial cells *in vivo* are quiescent cells that coat the ovarian surface at ovulation unless there is a growth stimulus such as that at the time of wound repair.

Follicular cells secrete epidermal growth factor, which then acts in a paracrine fashion to stimulate growth of epithelium after rupture of the follicle (Skinner *et al* 1987). EGF inhibits FSH-induced LH receptor expression and oestrogen production, but stimulates FSH receptor expression and FSH-induced progesterone production (Hiramatsu *et al* 1992), (Mondschein *et al* 1980, May *et al* 1987).

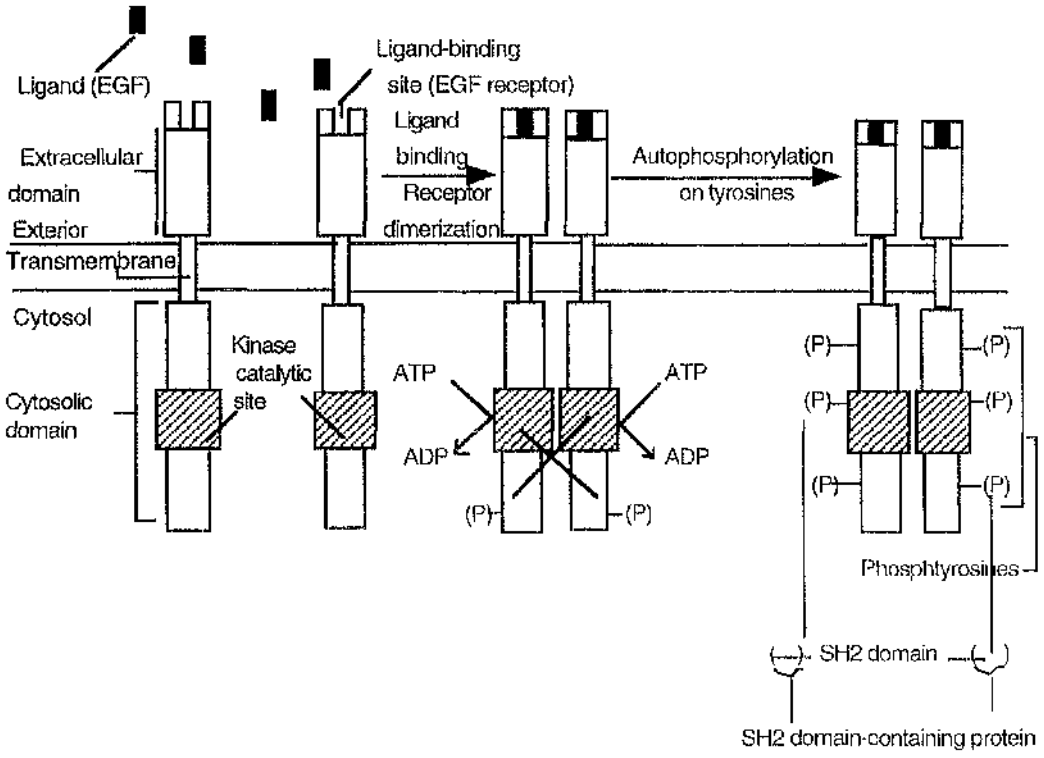
1.8.3 EGFR Expression in Normal Ovarian Epitheluim and Ovarian Cancer

Human ovarian epithelial cells normally express EGF-r and EGF may act to stimulate regrowth of epithelial cells after disruption of the ovarian surface at ovulation (Rodriguez *et al* 1991). This process could lead to the promotion of any transformed epithelial cells leading, eventually, to viable tumour formation.
The presence of EGF-r has often been detected in tissues derived from ovarian carcinomas (Bauknecht *et al* 1989, Kohler *et al* 1989). The EGF-r was present in 39.7% of samples ranging from 36.4% in those tumours which were classified as being mucinous to 47.7% in the undifferentiated group (Owens *et al* 1991). In the Bauknecht study EGF-receptor positive carcinomas were associated with a better survival rate of the patients. This may have been due to better response to chemotherapy in the EGFr+v patients. Normal ovarian epithelial cells express EGF receptor (10^4-10^5 receptors per cell) and EGF acts to stimulate proliferation of these cells (2-5 fold) (Rodriguez *et al* 1991). Ovarian cancer cell lines were found to express similar numbers of high-affinity EGF-receptors, but most of these cell lines were relatively resistant to the growth-stimulatory effect of EGF (Rodriguez *et al* 1991) and (Berchuck *et al* 1990).

1.8.4 Epidermal Growth Factor Receptor Tyrosine Kinase

Epidermal growth factor receptor (EGFr) is a 170 kD transmembrane glycoprotein that consists of an extra cellular domain that binds to EGF (Ullrich *et al* 1990). EGF binding activity of receptor preparations was shown to be associated with a protein kinase activity. Subsequent studies demonstrated that the EGF binding activity and kinase activity were properties of one and the same protein and that the kinase activity was a tyrosine phosphorylation (Cohen *et al* 1980).
In the absence of EGF the apparent tyrosine kinase activity is low. Binding of EGF to the receptor domain (figure 6) results in receptor dimerization and

Figure 6. Dimerization and activation of EGF receptor



tyrosine kinases is induced by ligand binding. EGF receptor contains an extracellular ligand-binding domain, a single transmembrane chain, which is an α-helix, and a cytosolic domain with tyrosine kinase activity. An activated receptor autophosphorylates specific tyrosine residues in the cytosolic domain. The resulting phosphotyrosines function as binding sites for various proteins containing SH2 domains. [Adapted from G. Panayotou and W. D. Waterfield. Bioassays 15: 171. 1993].

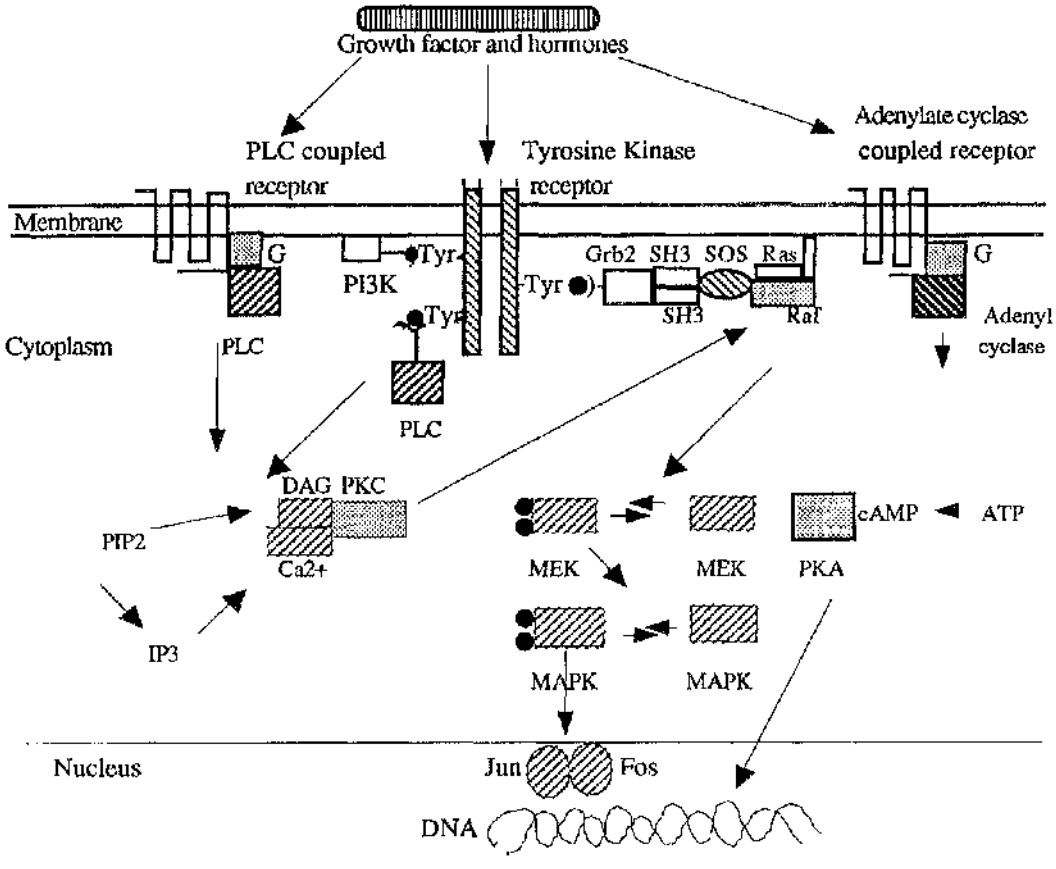
subsequent autophosphorylation of the cytoplasmic domain of EGFr at specific tyrosine residues (Downward *et al* 1984). This autophosphorylation results in an activation of the tyrosine kinase activity of the EGFr and can initiate the physiological effects of EGF by activating a variety of molecules such as phospholipase Cγ, phosphotidyl inositol 3 kinase and *ras* GTP*ase* activating protein (Margolis *et al* 1992, Ullrich *et al* 1990). Thus, over expression of EGFr and/or its ligands can result in rapid promotion of transformed cells when the receptor is functional. The evidence in favour of a role for EGFr is accumulating for a wide variety of epithelial cancers. Binding of protein tyrosine kinases occurs at highly specific phosphotyrosine residues of the receptor and is believed to be a mechanism for signal transduction from the receptor to the final intracellular target, including signal transduction to the nucleus (Lia *et al* 1994) see (figure 7). Enhanced RPTK activity resulting from tyrosine kinase over expression or amplification following persistent stimulation by autocrine secreted growth factors, can lead to disease (Bishop 1987). The activity of tyrosine kinase has been implicated in many cancers and non malignant proliferative diseases (Ross 1993).

Removal of phosphate groups from phosphorylated tyrosine residues by protein tyrosine phosphatases (PTPs) can down regulate the growth stimulatory activity of these signalling pathways (Wiener *et al* 1993). Dephosphorylation by tyrosine phosphatases class 1B which can block the ability of activated HER-2/*neu* to transform 3T3 cells (Brown-Shimer 1992).

Normal ovarian epithelium expresses very little of these phosphatases, but a majority of ovarian cancers express variable amounts of it (Wiener 1993).

The extra cellular domain of tyrosine kinase growth factor receptors can also be used as a target for treatment with monoclonal antibodies, either conjugated with toxins or unconjugated. Recent results with immunotoxins directed against C-*erb* B-2 are of particular interest in that only cells that over express the receptor are killed by the toxin (Rodriguez 1993).

Figure 7. Cell signalling pathways and MAP Kinases translocation to the nucleus



Abbreviations:
G, G protein; PLC, phospholipase C ;Tyr, tyrosineresidue; PI3K, phosphatidylinositol-3-kinase; Grb2, growth factor binding 2; SH3, src-homology region 3; sos, son-of-sevenless; MEK, MAP kinase kinase; MAPK, mitogen activated protein kinase; PIP2, phosphatiidylinositol 4,5 diphosphate; IP3, inositol (1,4,5)-triphosphate; DAG, diacylglycerol; PKC, protein kinase C; PKA, protein kinase A; ●), SH2 (src homology 2) domain. ● indicates phosphorylated form of kinase; Jun and Fos, transcription factors.

1.8.5 Transforming Growth Factor-α

Transforming growth factor-α (TGF-α), (a 48 amino acid polypeptide) interacts with the same receptor as EGF (Marquardt *et al* 1984). It is a regulatory molecule capable of affecting the same cell types, including epithelial cells that respond to EGF (Todaro *et al* 1980). There is substantial evidence for the synthesis of TGF-α during early fetal development but normal adult cells secrete little TGF-α (Lee *et al* 1985) and (Twardzik *et al* 1985). This suggests that TGF-α may function as an embryonic version of EGF.

The binding of either TGF-α or EGF to receptors on the surface of the target cells initiates a sequence of cellular responses culminating in cell division (Downward *et al* 1984) and (Ullrich *et al* 1984). Over expression of TGF-α in transgenic mice has been demonstrated to lead to neoplasias and carcinomas (Sandgren *et al* 1990) and (Jhappan *et al* 1990). These finding indicate that TGF-α may play some role in the pathogenesis of certain types of cancer.

1.9 Action of TGF-α in Ovarian Carcinoma Cell Lines

Expression of the EGF-r and secretion of TGF-α occurred in 16 of 17 cell lines derived from ovarian carcinoma. Stimulation of growth was observed after addition of TGF-α in 8 of 13 cell lines and inhibition of growth was noted after addition of neutralizing antibodies to TGF-α in 4 of 5 cell line (Kurt, Stromberg *et al* 1992). This result gives evidence that autocrine growth via TGF-α/EGF-r may be common in development and/or progression of ovarian carcinoma.

1.9.1 Involvement of TGF-α and EGFR in Primary Ovarian Cancer

EGF was detected in only 27.6% of ovarian cancers. TGF-α was present in 88.5% and is common in all histological sub-divisions (owens *et al* 1991).

A correlation between presence of EGF-r and expression of TGF-α has been observed (Arteaga *et al* 1988) and (Bauknecht *et al* 1990). Thus, it is possible these particular growth factors have an autocrine or paracrine role in the growth regulation of ovarian tumours. Antagonists to TGF-α/EGF-r autocrine mechanism could be effective in treating certain human ovarian cancers (Ken-Ichiro *et al* 1991).

1.9.2 Transforming Growth Factor-β

Transforming Growth Factor-β (TGF-β) a 25,000 mol wt homodimeric protein, was first isolated from media conditioned by transformed cells and identified as the protein responsible for the phenotypic transformation of marine fibroblasts (Delarco *et al* 1986). In addition to stimulating growth of fibroblastic cells in soft agar, TGF-β has a variety of actions including inhibition of epithelial cell growth (Massague 1990). TGF-β has been reported to modulate several ovarian functions such as FSH-dependent LH receptor induction in rat granulosa cells (Dodson *et al* 1987) and (Blair *et al* 1988). Three distinct molecular forms of TGF-β have been identified in mammals: TGF-β1, TGF-β2 and TGF-β3. It has been suggested that the development of some human carcinomas might be due to a loss of sensitivity to TGF-β (Roberts *et al* 1988).

The cellular effects of TGF-βs are multi-factorial and are dependent upon other factors, but in general, their effect upon epithelial cells is antimitotic (Sporn *et al* 1986). TGF-βs consist of two identical 112 amino acid chains synthesized as part of a larger, biologically latent peptide (Sporn *et al* 1986 ; Wakefield *et al* 1988). TGF-β can induce apoptosis in a fraction of ovarian cell lines and in tumour cells taken directly from patients (Havrilesky *et al* 1995).

TGF-β is secreted by thecal-interstitial cells from rat and bovine ovaries (Skinner *et al* 1987). TGF-β is a regulator of granulosa cell function *in vitro* (Bendell and Dorrington 1988). Ovarian cancer cell proliferation might be due to loss of down regulation by polypeptides of the TGF-β family, although little is known about a potential TGFβ1 autocrine loop in ovarian carcinoma cells (Berchuck *et al* 1990, Marth *et al* 1990). Retroviral transformation can result in greatly elevated levels of TGF-β mRNA and TGF-β synthesis (Derynck *et al* 1986) and (Anzano *et al* 1985). TGF-β inhibited proliferation of normal ovarian epithelial cells between 40 - 70% (Berchuck *et al* 1991). Each chain is synthesised as the C-terminal domain of a 390 amino acid precursor that has the characteristics of a secretory polypeptide (it contains a hydrophobic signal sequence for translocation across the endoplasmic reticulum and is glycosylated) (Derynck *et al* 1985) and (Purchio *et al* 1988). TGF-β appears to play a role in normal ovarian function, particularly in the regulation of granulosa cell functions in response to follicle stimulating hormone (Adashi *et al* 1989). The growth regulatory effects of TGF-β may be dependent upon the presence of specific classes of binding proteins (Roberts *et al* 1991). In addition, it has been shown that TGF-β is secreted in an inactive form non-covalently bound to a portion of its precursor, from which it must be released to produce the biologically active TGF-β.

Although TGF-β initially was discovered due to its ability to induce the transformed phenotype in some normal cells, its predominant effect on most normal epithelial cells, *in vitro* is inhibition of proliferation.

Expression of TGF-β is lost in a approximately 40% of ovarian cancers, but cells taken directly from ascites fluid can still be inhibited by exogenous TGF-β in 95% of cases (Hurteau *et al* 1994).

1.9.3 Oncogenes

An oncogene is a gene the protein product of which can cause cancer under particular circumstances. Many oncogenes were originally found in viruses which had hi-jacked the genes from animal hosts. Oncogenes usually code for proteins regulating cell division. In the last decade more than 50 genes have been discovered which can cause the transformation of cells, be they cultured or within the tissues of transgenic animals. These genes are dominantly transforming genes (Bishop 1987). The HER-2/*neu* (*c-erb* B2) proto-oncogene was first identified in studies in which NIH 3T3 cells were transformed with deoxyribonucleic acid from ethylnitroso urea-induced rat neuroblastomas (Padhy *et al* 1982).

The HER-2/*neu* oncogene encodes a 185 kd cell surface protein (P185) that has a cysteine-rich extra cellular domain, a membrane-spanning region, and an intracellular domain that has intrinsic tyrosine kinase activity (Schechter *et al* 1985) Over-expression of HER-2/*neu*, because of gene amplification, occurs in approximately 25% to 30% of breast and ovarian carcinomas (Slamon *et al* 1987) and (Slamon *et al* 1989). HER-2/*neu* may be a useful target for immunotherapy with unconjugated antibodies and immunotoxins in ovarian and breast cancers that over-express this proto-oncogene (Rodriguez *et al* 1993).

In contrast to oncogenes, there are a group of tumour suppressor genes whose protein products are essential to the elimination of transformed cells. Mutation and over-expression of the p53 gene is the most frequent genetic change to be described in human cancers so far (Nigro *et al* 1989). Over-expression of p53 has been observed in 50% of ovarian cancers and allelic deletion at the p53 locus in 25-55% of epithelial ovarian cancers, also loss of heterozygosity on 17q occurs in some sporadic ovarian cancer (Eccles *et al* 1990) and (Sato *et al* 1991).
In each of 9 cases studied from 17q-linked breast/ovarian cancer families, the allele losses affected the wild-type chromosome, consistent with a retinoblastoma like model in which the predisposing gene is a suppressor gene (Smith *et al* 1993).

1.10 Aims and Objectives of Thesis

As can be seen from the preceding literature survey, onset of ovarian cancer may be the result of promotion of transformed cells during post-ovulatory wound repair, driven by the EGF/TGFα system.

Alternatively, it may be due to loss of sensitivity to TGFβ or, indeed, to some combination of the two. Additionally, previous work from our laboratory has shown that the EGFr-associated tyrosine kinase may become elevated in platinum resistant ovarian cancer cells.

As an in vitro approach to both developing new anti-ovarian cancer therapies and understanding more of the biology of this disease, this thesis examines:

1. The regulation of ovarian cancer cell growth by EGF/TGFα.

2. The inhibition of growth by selective blocking of the EGFr-associated tyrosine kinase.

3. The role of TGFβ in mediating the growth inhibitory effect of LH.

4. The relative effects of EGF/TGFα and LH on the morphology of ovarian cancer cells.

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Growth Media

Dulbecco's modification of Eagle's medium (RPMI 1640) was originally supplied by Gibco. To eliminate the oestrogenic action of phenol red within the culture this was replaced by phenol red-free RPMI powder obtained from Sigma.

2.1.2 Supplements to Cell Growth Media

All other cell culture media and supplements were obtained from Gibco.

2.1.3 Cell Culture Materials

Biofreeze Vials	Nunc (Denmark)
Dialysis Membrane	Med International Ltd
25,75, 175 cm^2 T/C flasks	Nunc
24 well FB plates	Corning
Nalgene (Bottle Top Filter)	Nalge (Europe) Ltd
Sterile Acrodisc (0.2μm Syringe Filters)	German Sciences
Tissue Culture Chambers	Nunc

2.1.4 Fine Chemicals

All chemicals used were of AnalaR grade or equivalent and were obtained from the following:

Bacitracin	Sigma Chemical Co. Ld
Bovine Serum Albumin	Sigma Chemical Co. Ld
Cycloheximide	Sigma Chemical Co. Ld
Dextran	Sigma Chemical Co. Ld
Dimethylsulphoxide	May and Baker
Dithiothreitol	Sigma Chemical Co. Ld
EDTA	Fisons

2.1.5 Miscellaneous Materials

DNA (calf thymeus) type XV	Sigma Chemical Co. Ltd
Hoechst 33258	Sigma Chemical Co. Ltd
Protein assay dye reagent concentrate	Bio-Rad
RibonucleaseA	Sigma Chemical Co. Ltd
Scintillation fluid (Ultima-Flo)	Packard
ZM (252868)	Zeneca

Suppliers for any other specific materials or apparatus are indicated throughout the text.

2.1.6 Hormones/Growth Factors

Gonadotrophin (LH)	Sigma Chemical Co. Ld
Gonadotrophin (FSH)	Sigma Chemical Co. Ld
Marine Epidermal Growth Factor	Biogenesis
Oestrogens and Progesterone	Sigma Chemical Co. Ld
Transforming Growth Factor-α	Sigma Chemical Co. Ld
Transforming Growth Factor-β1	Sigma Chemical Co. Ld

Transforming Growth Factor-β-receptor	Sigma Chemical Co. Ld
TGF-β1 Monoclonal antibody (TB21)	Bionostics Limited

2.1.7 EGF-receptor Study Reagents

EGF-receptor tyrosine kinase assay kit	Amersham
EGF-receptor Assay kit	Vienna Lab

2.2 BUFFERS AND SOLUTIONS

2.2.1 Buffers

2.2.2. Phosphate Buffered Saline (PBS) pH7.2

170 mM NaCl

1 mM $Na2HPO_4$

2 mM KH_2PO_4.

2.2.3 ETN Buffer

10 mM EDTA

10 mM Tris-HCL

100 mM Sodium Chloride, pH 7.

2.2.4 Hank's Modified Buffer

Calcium Chloride 1.3 mM

Potassium Chloride 5.4 mM

Magnesium Chloride 0.5 mM

Magnesium Sulphate 0.5 mM

Sodium Chloride 137 mM

Sodium hydrogen carbonate 4 mM

Sodium dihydrogen orthophsphate 0.4 mM, pH 7.2 - 7.4

2.2.5 HE Buffer pH 7.4

20 mM HEPES
1.5 mM EDTA
10% Glycerol (v/v)

2.2.6 HED Buffer pH 7.4

HE Buffer plus 0.25 mM Dithiothreitol (DTT)
This was made freshly on the day of use.

2.2.7 HEPES Buffered Saline pH 7.4 (HBS)

10 mM HEPES, 150 mM NaCl

2.3 CELL CULTURE SOLUTIONS

2.3.1 Routine Sub-Culture Medium

RPMI 1640 medium with L-Glutamine; 10% (v/v) Fetal Calf Serum (FCS); 60 mg/ml Tylocine; 100 units/ml Penicillin; 50mg/ml Streptomycin.

2.3.2 Dextran-Coated Charcoal

100 ml sterile PBS-A
0.25% charcoal = 0.25g
0.0025% dextran = 0.0025g
The mixture was stirred for 30 minutes, dispensed into 25 ml

bottles and centrifuged. The supernatant was discarded and dialysed serum added to the pellet see section 2.3.3.

2.3.3 Dialysed Heat-Inactivated Dextran-Coated Charcoal Stripped Serum (DHIDCCFCS)

100 mls of FCS was dialysed against four 1 litre changes of Hank's modified buffer over 48 hours at 4°C. Following dialysis the serum was transferred to a glass container and heat inactivated for 45 mins at 56°C. The serum was then cooled to 4°C and added to a pellet of dextran coated charcoal (0.25% Charcoal w/v, 0.0025% Dextran w/v in 25 ml PBS-A which had been centrifuged for 30 min and the supernatant removed). This solution was allowed to stir at 4°C for 30 min. The dialysed serum was centrifuged for 30 min at 10,000 g, the supernatant was filter-sterilised using 0.2 micron filters and stored at -20°C

2.3.4. Dialysing Tubing Preparation

Approximately 50 cm strips were boiled for 15 minutes in 0.2% (w/v) sodium bicarbonate. After 15 minutes, the strips were rinsed in distilled water and stored in 50% methanol.

2.3.5 Versene

125 mM Sodium Chloride
2.7 mM Potassium Chloride
6.3 mM Di-Sodium hydrogen orthophosphate
3.2 mM Potassium dihydrogen orthophosphate
0.5 mM EDTA.

2.3.6 Trypsin/Versene

40 ml Versene : 10 ml 0.25% (w/v) Trypsin Solution

2.3.7 Cell Culture Freezing Medium

RPMI 1640 medium with L-Glutamine; 10% FCS (v/v); 10% Dimethyl Sulphoxide (v/v).

2.4 CELL CULTURE METHODS

2.4.1 Cell Lines

OAW42 ovarian cancer cell line model (Wilson *et al* 1984) was kindly supplied by Dr. Anne Wilson (Derby). This cell line was derived from the malignant epithelial cells of a metastatic ovarian tumour.

PEO1 and PEO1cddp ovarian cancer cell lines were derived from malignant effusions from a single ovarian cancer patient before and after her tumour became refractory to platinum therapy (Langdon *et al* 1988) was kindly supplied by Dr. Simon Langdon (ICRF, Edinburgh).

2.4.2 Routine Cell Culture

All cell lines were routinely grown at 37°C in a 5% CO_2 atmosphere. All aseptic manipulations were performed within the confines of a laminar air flow cabinet (Flow Labs). Initially routine subculture was performed using Dulbecco's modification of Eagle's medium (RPMI 1640) supplemented with 10% fetal calf serum (FCS), HEPES, pH 7.4 (10 mM), penicillin (100 units/ml), streptomycin (50 mg/ml) and Glutamine 10% (v/v). Cell medium was changed every two to three days. Routine subculture was carried out using RPMI 1640 supplied as a powder supplemented with 5% dialysed HIDCCFCS. All routine and experimental media were changed every 48-72 hours.

2.4.3 Subculture Technique

Stocks of cells were grown routinely in 175 cm^2 plastic tissue culture flasks. After reaching 80-90% confluence,the medium was removed and the cells washed twice with 20 ml PBS at 37°C. 0.05% trypsin/versene solution (prewarmed to 37°C) was added to the culture flasks; 0.2 ml per well of a 24 well plate,1 ml per 25 cm^2 flask, 3 ml per 75 cm^2 flask and 8 ml per 175 cm^2 flask. The cells were incubated at 37°C in trypsin solution for 2-4 minutes. The trypsinization reaction was stopped by adding 2 volumes of fresh medium (37°C) followed by rapid pipetting to ensure an even cell suspension. The cell suspension was then dispensed into 3-5 sterile culture flasks to which growth medium had been added before a final rapid pipetting ensured even distribution of the cells. Seeding of experimental plates and flasks was carried out using the technique described above with the following differences.

After the trypsinization was stopped, the cell number per ml was counted using a haemocytometer and the suspension was diluted to the required concentration with routine medium. After 24-48 hours the routine medium was removed and the cells washed twice with prewarmed PBS before the experimental media and supplements were added. Initial plating was designed to achieve about 25% confluence and cell numbers were $5x10^4$ cells/well of a 24 well plate, $5x10^6$ cells/25 cm2 plats and $5x10^3$ cells/well of a 96 well plates.

2.4.4 Mycoplasma Testing of Cell Lines

Mycoplasmas are a common and serious contamination of cell culture which can not be detected with the naked eye (Russel, 1975). They cause changes in metabolism, growth and viability which lead to variability of results. Routine mycoplasma testing is therefore important to ensure early detection and treatment of any infection. An immunoassay mycoplasma detection kit (Boehringer Mannheim) was used for detection.To increase the sensitivity of the assay; the cells

were incubated in antibiotic-and anti-PPLO-free medium for 72h prior to the immunoassay being carried out. Firstly, microtiter plates were coated with antibodies to one of the four commonest mycoplasma strains (*Mycoplasma arginini*, *M.hyorhinis*, *A.laidawii*, *M.orale*) by incubating plates for 2h at 37°C in coating solution. Nonspecific binding sites were blocked and the plates washed before cell samples and controls were added and incubated overnight at 4°C. Detection was carried out by incubating plates for 2h at 37°C with biotin-conjugated antibodies against the mycoplasma strains mentioned above, followed by a 1h incubation with streptavidin-AP and a final 1h incubation with 4-nitro-phenylphosphate. Evaluation was carried out visually, a mycoplasma positive plate being yellow in colour whereas a mycoplasma negative plate is colourless. This procedure was carried out simultaneously on all the cell lines used in the laboratory every three months. If any cell lines were infected all the growing cells were destroyed, as was any opened medium, supplement, buffer or plastic ware.
The cell culture unit and incubators were cleaned with Decon and 70% ethanol prior to fresh frozen stocks (frozen down prior to the last totally negative mycoplasma testing) being defrosted and seeded down. To help prevent mycoplasma infection, all medium was supplemented with anti-PPLO.

2.4.5 Preparation of Frozen Cell Stocks

To ensure that all experiments could be carried out on cells of a similar passage number and to protect against loss of a cell line due to contamination, frozen stocks of cells were prepared. One 180 cm^2 flask of cells at about 70-80% confluence was trypsinized as in section (2.4.3) and the reaction was stopped by adding 10 ml of routine medium. The cell suspension was transferred to a sterile universal container. The cells were pelleted by centrifugation at 1000g for 2 minutes at 4°C and the supernatant removed. The cells were resuspended in 4 ml of freezing medium as in section (2.3.7) and 1ml volumes were transferred to sterile Biofreeze vials prior to being frozen at -70°C. They were then stored under liquid nitrogen.

2.4.6 Recovery of Frozen Cells

To grow cells from frozen stocks the vials were thawed by placing in 37°C water bath for 5-10 minutes. The vials were removed from the water bath before all the ice had melted, and the cell suspension was transferred to a universal container to which 10 ml of routine medium (37°C) had been added. The suspension was then centrifuged for 2 minutes at 1000g before discarding the supernatant and resuspending the cells in medium at 37°C. The cell suspension was placed in a 75 cm^2 flask and incubated at 37°C, as described in section (2.4.2).

2.4.7 SDS Lysis Buffer

The cell membrane had to be ruptured before many of the assays and procedures could be carried out. In the first method the cells were trypsinised as in section (2.4.3) and 10 ml of routine medium was added. The cell suspension was transferred to a sterile universal container.
This was then centrifuged for 2 minutes at 1000g before discarding the supernatant and resuspending the pellet in 0.2% SDS in ETN buffer section (2.2.3) followed by incubation at 37°C for 30 minutes. This technique solubilises the DNA and denatures proteins, as well as solubilising the membranes.

2.5 EXPERIMENTAL METHODOLOGY

2.5.1 DNA Synthesis Experiments

Cells were plated down for 24 h in RPMI + 10% FCS in 24 well F.B. plates. This medium was removed and the cells washed with two changes of sterile PBS-A (1ml). Experimental medium (Phenol Red Free Medium) + 5% dialysed HIDCCFCS, with or without hormones and growth factors were added to a final volume of 1 ml per well. At appropriate incubation times, wells were harvested by the removal of the medium and washing twice with PBS-A (1ml). To each well

200µl aliquots 0.1% (w/v) SDS in ETN buffer were added. Samples were solubilised by incubation at 37°C for 30 minutes and stored at -20°C until the day of DNA assay.

2.5.2 Hoechst DNA Assay

The assay methodology is essentially that described by Leake & Habib (1987) and involves the intercalation of Hoechst 33258 into solubilised DNA resulting in a complex which fluoresces with a maximum emission at 450 nm. Solubilised samples were transferred to RT-30 tubes, and to each tube, 3 ml of ETN buffer containing Hoechst 33258 (100 ng/ml) and RNAase (5 µg/ml) was added. The tubes were then vortexed and incubated at 37°C for 15 minutes in the dark. Fluorescent enhancement at 450 nm was measured using a Hitachi-Perkin Elmer MPE-2A fluorescence spectrometer at an excitation wavelength of 360 nm with both slit widths set at 5 nm.
The results obtained were compared to a standard curve range of calf thymus DNA of 0-50 µg/ml see section (2.5.4).

2.5.3 Preparation of Samples for Hoechst DNA Assay

Aliquots of cell suspension were centrifuged at 1000*g* for 5min at 4°C. The supernatant was discarded and 200 µl of 0.1% (w/v) SDS in ETN buffer added. The pellet was solubilised at 37°C for 30 mins. DNA was estimated using the standard curve (2.5.2).

2.5.4 Hoechst DNA Standard

DNA standards were prepared from a 100µg/ml solution of calf thymus DNA dissolved in ETN buffer which was diluted to give standard solutions containing 5,

10, 15, 20, 30, 40, and 50µg/ml calf thymes DNA. The concentrations of these solution were verified by measuring their absorbances at 260nm as 1unit of absorbance at this wavelength corresponds to 50 µg/ml DNA. The standards were stored for up to a month at -20°C.

2.6 BRADFORD PROTEIN ASSAY

2.6.1 Protein Standard

Bovine serum albumin (BSA) was used to create protein standard solutions ranging from 0-500 µg/ml. This was achieved by diluting a stock of 1mg/ml BSA with PBS or the same buffer as the samples to create standards of 0, 10, 20, 50, 100, 200, and 500 µg/ml protein content. The standards were stored at 4°C for up to two weeks.

2.6.2 Assay Protocol

This assay is based on the principle that there is a shift in maximum absorbance from 465 nm to 595 nm when protein binds to a solution of Coomassie Brilliant Blue G-250 (Bradford, 1976). Cells were lysed using the SDS lysis method (section 2.2.6). Dilutions of the samples were made (1:10 and 1:100). Biorad Bradford reagent was prepared by diluting commercial stock 1:6 and filtering through Whitman filter paper No. 1. The assay was carried out by placing 0.2 ml of each sample and standard into RT-30 tubes in duplicate and adding 0.8 ml of Bradford reagent. These were mixed gently and incubated at room temperature for 30 minutes before the absorbance was read at 595 nm using a spectrophotometer (LKB ultra spec 4050). The absorbance of each standard was then plotted against the protein concentration of the standards to create a calibration curve, which was used to calculate the protein content of each of the samples.

2.7 MTT ASSAY

MTT is a yellow water soluble tetrazolium dye that is reduced by live, but not dead, cells to a purple formazan product which is insoluble in aqueous solution. It can be used as a measure of cell viability (Mossmann, 1983) However, a number of factors can influence the reduction of MTT (Vistica *et al* 1991).

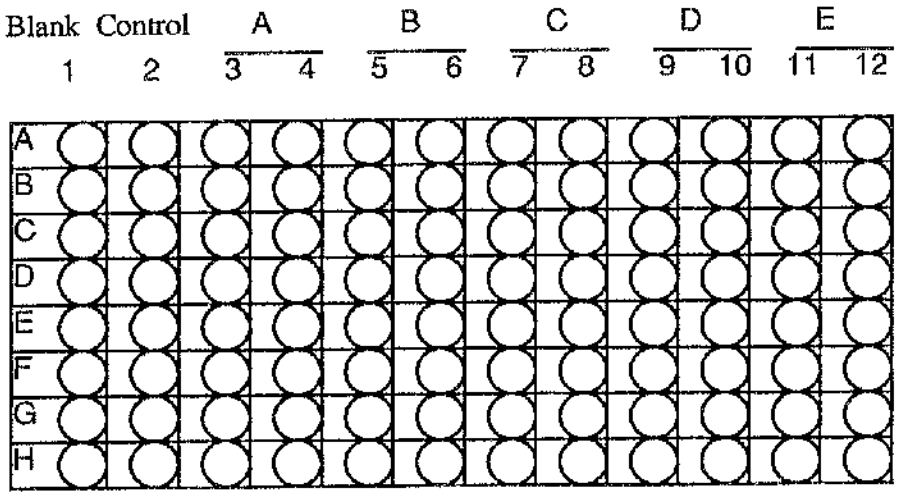
2.7.1 Experimental Protocol

2.7.2 Plating Down Cells

A sub-confluent monolayer culture was trypsinized and cells were collected in growth medium containing serum.

The cell suspension was centrifuged (200g, 5 minutes) to pellet cells. These were resuspended in growth medium and cell number counted using haemocytometer. Cells were diluted to a density of 2.5-50x 10^3 cells/ml, depending on the growth rate of the cell line. Cell suspensions were transferred to 10 cm^2 petri dishes using a multichannel pipette; 200 µl of suspension was added into each well of columns 2-12 of a flat bottomed 96 well tissue culture plate (i.e. 88 wells per plate contained cells) starting with column 2 and ending with column 12 (see figure 8). 200 µl of growth medium was added to the 8 wells in columns. Plates were incubated in a humidified atmosphere at 37°C for 24 hours such that cells were in the exponential phase of growth at the time of replacing medium with that containing hormones, growth factors etc.

Figure 8. Diagram of 96 well tissue culture plate



2.7.3 Estimation of Surviving Cell Numbers

At the end of the exposure period to experimental additives, the medium was removed from all wells containing cells and the cells were fed with 200 µl of fresh medium. 50 µl of MTT (5 mg/ml in sterilised PBS) was added to all wells in columns 1 to 12.

Plates were wrapped in aluminium foil and incubated for 4 hours in a humidified atmosphere at 37°C. This was a minimum incubation time and plates can be left for up to 8 hours. The medium and MTT were then removed from the wells and the MTT-formazan crystals were dissolved by addition of 200 µl of DMSO to all wells in columns 1 to 12. Glycine buffer (pH 10.5) was added (25 µl per well) to all wells. Absorbance was recorded at 540 nm immediately since the product was unstable. The wells in column 1, which contained medium, MTT but no cells were used to blank the plate reader.

2.7.4 Analysis of Results

A graph was plotted of absorbance (Y axis) against concentration (X axis). The mean absorbance reading from the 8 wells in column 2 was used as the control.
Any variations among the readings for these 8 wells reflect differences in initial plating of cells and/or growth conditions across the plate. By taking the mean value for each set of 8 wells, such variations should be eliminated.

2.8 EPIDERMAL GROWTH FACTOR RECEPTOR ASSAY

The Vienna Lab EGF receptor Assay (Vienna Labs, Vienna, Austria) provided materials for the quantitative measurement of Epidermal Growth Factor receptors (EGFr) in cell membrane fractions from cancer cell lines or human tumour tissues (e.g. breast, ovarian carcinoma). This kit has been carefully evaluated in several laboratories and shown to give exactly comparable quantitative results as the EORTC standard Ligand Binding Assay for EGFr (Oberkanins *et al* 1995).

2.8.1 Components of the EGF-R "Scatchard" Assay

Assay Buffer	(40ml)
Storage Buffer	(2x concentrate; 20ml)
Tracer Dilution Buffer	(15ml)
[^{125}I]-EGF	(lyophilized)

The lyophilised ^{125}I-EGF was reconstituted by adding 2.5 ml Tracer Dilution Buffer. Subsequent dilutions (into the colourless tubes) allow assay over a final concentration range of 0.1-4 n molar.

The kit also contained :

Reaction Tubes	(colourless; 110 tubes)
Reaction Tubes with EGF	(green label; 30 tubes *)

* Tubes contain lyphilized EGF resulting in a 200x excess over [^{125}I]-EGF at 1 nM during incubation to allow determination of non-specific binding.

Control (1 vial)

The Control contains lyophilized human placenta membranes. These were reconstituted by adding 1 ml Assay Buffer (0-4^{o}C) immediately prior to analysis.

Precipitation Reagent 1 (45 ml)

This was shaken well before use

Precipitation Reagent 2 (45 ml)

Precipitation Reagent 3 (concentrate)

This was prepared as a working solution by adding H_2O to a final volume of 600 ml.

2.8.2 Processing of Cell Samples

Cells (70-80% confluent), grown in 175 cm^2 plastic flasks in routine medium were harvested and pelleted by centrifugation at 1000 g for 5 mins. The cell pellet was then washed twice in ice-cold PBS-A and resuspended in 4 ml of HED buffer (10 mM Hepes, 1.5 mM EDTA, 0.5 mM Dithiothreitol, pH 7.4).

2.8.3 Cell Homogenization

Cells were mixed with a suitable homogenization buffer (10 mM Hepes, 50 mM sodium chloride, 0.1% (w/v) BSA, pH 7.0). Cells were homogenized using Ultra-turrax homogeniser followed by gently homogenization using a glass/glass tissue grinder for 2x10 second bursts to ensure an even suspension. The temperature of the suspension was not allowed to rise above 8°C at any time.

2.8.4 Separation of Nuclei, Membranes, and Cytosol

Cell homogenates were centrifuged at 800 g for 15 min at 4°C to collect cell debris and nuclei. The supernatant was further centrifuged at 50,000 g for 60 min at 4°C. The resulting supernatant (cytosol) was transferred into a fresh container.

The pellet containing cellular membranes was resuspended in 400-500 µl assay buffer. A homogeneous suspension was achieved by repeatedly pipetting through the tip of a micropipette.

The volume of the suspension was determined by the pipette. An equal volume of storage buffer was added (provided as a 2x concentrate).

An aliquot (25-50 µl) was removed for protein measurement. The membrane suspension was stored at -20°C.

2.8.5 Preparation of Reagents and Samples

The reaction tubes were allowed to warm up to room temperature (at least 30 min). Two green tubes for each sample to be analyzed were removed.

The package was tightly resealed and the remaining tubes were stored at 4°C.

A working solution of precipitation reagent 3 was prepared by dilution with H_2O, to a total volume of 600 ml. Precipitation reagents 1,2 and 3 were kept at 0-4°C.

The lyophilized [^{125}I]-EGF ("Tracer") was dissolved in 2.5 ml Tracer dilution buffer at 0-4°C. Dilutions of this Tracer stock solution were prepared according to the following scheme :

"Tracer" Dilution	C1	C2	C3	C4	C5	C6	C7	C8
Final [^{125}I]-EGF Conc	0.10	0.25	0.50	0.75	1.00	1.50	2.50	4.00nM
Tracer stock solution	20	50	100	150	500	300	500	800 µl
Tracer Dilution Buffer	1180	1150	1100	1050	2500	900	700	400 µl

Tracer dilutions C1-C8 were kept refrigerated (0-4°C) while performing the assay.

The lyophilized control was resuspended in 1 ml assay buffer and carefully mixed

by pipetting several times, and kept on ice until starting incubations with tracer dilution.

Samples were diluted with assay buffer to a concentration range of 0.3-1.5 mg membrane protein per ml. A minimum of 750 µl of each diluted sample was required for saturation analysis. Samples were mixed carefully by pipetting several times, for homogeneity. Diluted samples were kept on ice until starting incubations with tracer dilutions.

2.8.6 Pipetting Scheme

Eight colourless tubes were prepared (to determine "total binding") (Tubes to receive C1-C8 as described on the previous page) and two tubes with green label (to determine "unspecific binding") (each containing 1n molar ^{125}I-EGF + 200-fold unlabelled EGF) for each sample to be analyzed.

75 µl of each sample was dispensed into the eight colourless tubes and the two green tubes.

75 µl of each [^{125}I]-EGF ("Tracer") dilution was added to the appropriately labelledtubes.

75µl of tracer dilution C5 (final concentration 1n molar ^{125}I-EGF) was added to the tubes marked green.

2.8.7 Incubation and Separation

Tubes were shaken gently and incubated at room temperature for 2 hours.

Whilst the experimental tubes were incubating, 3 different tubes for each final tracer

concentration C1-C8 were counted in a gamma-counter for 1 min to determine cpm "Total counts (C1-C8)".

Incubation was terminated by adding 300 µl precipitation reagent 1 to all tubes. (reagent was mixed by vortexing before use).

300 µl precipitation reagent 2 was added to all tubes and mixed by vortexing.

After 10 min, 2 ml precipitation reagent 3 was added to all tubes and mixed.

Tubes were centrifuged at 3.000 xg for 15 min at 4°C.

Supernatants were decanted (Tubes were placed in a tightly fitting foam rack which allowed multiple supernatants to be decanted simultaneously).

Pellets were washed by adding 2 ml precipitation reagent 3 to all tubes, briefly vortexed and centrifuged at 3.000xg for 15 min at 4°C.

Supernatants were decanted and tubes were drained by standing them inverted on paper towels for 1-3 min, pellets were counted in a gamma-counter for 1 min.

2.8.8 Calculation of Results

To calculate maximum binding capacity and dissociation constant of EGFr in a sample the following parameters have to be known:

Total Binding (BT)
cpm of pellets in colourless tubes (1 tube per sample for each final Tracer concentration C1-C8)

Unspecific Binding (BU)
cpm of pellets in tubes marked green (mean of 2 tubes per sample)

Total Counts (T)
cpm of tubes before adding precipitation reagents (mean of 3 tubes for each final Tracer concentration C1-C8)

Protein Concentration

The protein concentration of the diluted sample which was incubated with Tracer dilution ([mg/ml] the respective value must be between 0.3-1.5)

The Maximum Binding Capacity of the membrane preparation as "fmol EGFr per mg membrane protein".

$$\frac{\text{x-intersection [fmol/tube]}}{\text{Protein conc. [mg/ml]x 0.075}} = \text{fmol EGFr/mg membrane protein}$$

2.9 EGF RECEPTOR TYROSINE KINASE ENZYME ASSAY

Binding of EGF to the receptor domain results in receptor dimerization and autophosphorylation and an activation of the tyrosine kinase activity of EGFr (Downward *et al* 1984). The phosphorylated tyrosine residues of the EGFr are involved in binding enzymes containing SH2 or SH3 domains. Enzymes that bind to EGFr phosphotyrosine residues include phosphatidyl inositol 3 kinase, phosphlipase Cγ and ras GTPase activating protein (Margolis *et al* 1992 and Ullrich *et al* 1990).

2.9.1 Substrate Buffer

A ready to use solution of 1.5 mM peptide in a buffer containing 135 mM HEPES, 300 μM sodium orthovanadate, 3 mM dithiothreitol, 0.5% Triton X-100, 6% glycerol and 0.05% sodium azide pH7.4

2.9.2 Magnesium ATP Buffer

A ready to use solution of 1.2 mM ATP in a buffer containing 30 mM Hepes, 72 mM magnesium chloride and 0.05% sodium azide pH7.4.

2.9.3 Epidermal Growth Factor (EGF)

Human recombinant epidermal growth factor (6μM).

2.9.4 Stop Reagent

A ready to use solution of 300 mM orthophosphoric acid containing carosine red.

2.9.5 Peptide Binding Papers

Twenty sheets of peptide binding paper containing 12 assay discs (3.0m diameter) allowing 240 assay to be performed. Each disc is individually numbered.

2.9.6 Sample Preparation

Samples of tissue or cells were homogenized in buffer containing 50mM Tris, 1mM $MgCl_2$, 2mM EDTA, 20 μg/ml soybean trypsin inhibitor and 50μg/ml PMSF (the latter two as protease inhibitors). A suitable buffer for solubilization and storage is 50 mM Hepes, 20% glycerol, 1% Triton X-100, 0.1% BSA, 0.05% sodium azide. Storage of solubilised EGFr TK at -80°C was recommended by Amersham. Apparent low values, obtained for concentrated samples may be due to interfering effects of some reagents used for sample preparation and competing enzyme activities in the sample (ATP hydrolyzing and proteolytic enzyme).

The Kit manufacturer recommended that dilution may reduce or obviate these effects. Sample were diluted and assayed as described (2.9.9). Calculation of total apparent EGFr TK activity was obtained from consideration of the dilution factor and the extent of phosphorylation using the formula described on (2.9.11).

2.9.7 Incubation Conditions

The assay is designed to measure the incorporation of phosphorus-32 into peptide at 30°C for a period of 30 minutes. The assay was linear for incubation times of up to 45 minutes and incorporation of up to 20% of total ATP. Further incubation or incorporation may not be a linear and so was avoided.

2.9.8 Reagent Preparation

All assay components were thawed and warmed to 30°C before beginning the assay Once thawed, each was reconstituted thoroughly by swirling and inversion and placed on ice. Unused reagents were re-frozen and stored at -15 to -30°C.
The magnesium [^{32}P] ATP buffer was prepared using a minimum of 9.25 KBq, 0.25μCi, of [^{32}P] ATP per tube. Sufficient reagent 3 was dispensed to perform the number of assays required.

2.9.9 Assay Protocol

1. The reagents were prepared as described previously.
2. Tubes were labelled (in duplicate) for samples and placed in a rack.
3. 10 μl of sample or buffer alone were pipetted into each appropriate tube.
4. 5μl of EGF were pipetted into each tube, or 5μl water for no EGF control blanks
5. 10 μl of substrate buffer were pipetted into each tube.
6. The reaction were starting by adding 5 μl of magnesium [^{32}P] ATP buffer.

The contents of the tube was mixed.

7. The tubes were incubated at 30°C for 30 minutes. During the incubation time, the binding discs were prepared. The required number of discs were cut out without removing discs from the sheet and placed in a dry tray suitable for washing the papers.

8. 10 µl stop reagent was added to each tube to terminate the reaction.

9. The terminated reaction tubes were shaken. The tubes were microfuged for 15 seconds to wash all reagent into base of the tube.

10. The phosphorylated peptide was separated as described in section (2.9.10).

2.9.10 Separation Procedure

Paper discs were provided in sheets of 12 disc. 30 µl of terminated reaction mixture were pipetted into the centre of each paper disc. Each paper of 12 discs was washed as follows:

1. 250 ml 75 mM orthophosphoric acid or 1% acetic acid were added to the wash tray containing the paper discs, not more than 25 discs per wash tray.
The tray was rocked by hand for 2 minutes. Wash reagent was decanted and disposed of as phosphorus-32 liquid waste. A similar volume of wash reagent was added and the wash repeated once.

2. 250 ml of distilled water were added to each wash tray. The tray was rocked gently by hand for 2 minutes. Water was decanted and disposed of as phosphorus-32 liquid waste. A similar volume of water was added and the wash repeated once.

3. 200 ml of water was added to the wash tray. The tray was rocked gently to float the paper discs. Using forceps, each paper disc was removed from the paper sheet and the disc was placed in a 20 ml scintillation vial. Water was disposed of as phosphorus-32 liquid waste and the paper sheet as phosphorus-32 solid waste.

4. 10 ml scintillant was added to each vial and was counted in an appropriate scintillation counter for phosphorus-32.

2.9.11 Calculation of Results

The phosphorus-32 incorporated into the peptide was quantitatively measured as follows. In the presence of enzyme sample and EGF the [^{32}P] counted on the papers was the sum of non specific [^{32}P] ATP binding, specific binding of phosphorylated peptide and binding of phosphorylated proteins in the cell extract (A). In the absence of EGF and presence of enzyme, the [^{32}P] counted on the papers was the sum of non specific [^{32}P] ATP binding, and non EGF dependent tyrosine kinase phosphorylation of the peptide and cell extract proteins (B).

In the absence of enzyme and presence of EGF the [^{32}P] counted on the papers is due to non specific binding of [^{32}P] ATP or its radiolytic decomposition products(C). EGF dependent tyrosine activity (EGFr TK) is therefore obtained from (A)-[(B)+(C)].

Calculation of specific activity (R) of 1.2 mM magnesium [^{32}P] ATP:

$$R = \frac{\text{cpm 5µl Mg[}^{32}\text{P]ATP}}{6} \text{ cpm/nmole}$$

Calculation of total phosphate (T) transferred to peptide and endogenous proteins 30 µl spotted onto binding paper.

Total terminated volume 40 µl. T= (A- [(B) + (C)]) X 1.33.

Calculation of pmoles phosphate (P) transferred per minute

$$P = \frac{T \times 100}{I \times R} \text{ p moles/minute}$$

where I = incubation time (min).

2.10 ELECTRON MICROSCOPIC PROCEDURE

OAW42 cells were studied for morphological change using Electron Microscopy. The cells were fixed for 1 hour using 2% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2 (Sabatini *et al* 1963). The specimens were then given three 20 minutes washes in 0.1M sodium cacodylate buffer at pH 7.2. Post-fixation was carried out using 1% osmium tetroxide in 0.1M sodium cacodylate buffer at pH 7.2. Following fixation the specimens were dehydrated with graded alcohol and embedded in Araldite (Glauert 1991). Ultrathin sections were cut with a Diatom diamond knife and mounted on formvar coated 1000 μm aperture grids. The ultrathin sections were double stained with uranyl acetate (Stempak *et al* 1964) and lead citrate (Reynolds 1963). The stained sections were examined using a Zeiss E.M. 109 electron microscope at 50 kV and electronmicrographs taken on Ilford FP4 film.

2.11 TGF-B1 WHOLE CELL BINDING ASSAY

These experiments were performed to estimate the number and binding affinity of TGF-β receptors on the OAW42 cell surface. These ovarian epithelial cells were seeded in routine medium onto a 24 well plate at initial density of 5 x 10^4 cells/well and incubated at 37°C overnight in a 5% CO_2 atmosphere.

Cells were washed three times with sterile PBS-A followed by addition of RPMI medium containing 0.1% PBS-A and grown until almost confluent. The cells were then washed in three changes of sterile PBS-A /0.1% BSA. Cells were harvested from eight wells for protein and DNA assay.

To each well 200 µl aliquots 0.1% (w/v) SDS in ETN buffer was added.

Samples were solubilised by incubation at 37°C for 30 minutes. Solubilised samples were transferred to eppendorf tubes and stored at -20°C until the day of DNA and protein assay.

To eight further wells, 200 µl binding buffer (*) containing 100 pM ^{125}-I-TGF-β1 were added. To another eight wells, 200 µl binding buffer containing 100 pM ^{125}-I-TGF-β1+20 nM TGF-β1 (unlabelled) were added.

Cells were incubated at 25°C for 2 hours. Binding buffer was aspirated off and disposed as radioactive waste. Cells were washed four times with ice-cold PBS/0.1% BSA.

0.1% SDS (500 µl) was added to each well, followed by incubation at 37°C for 30 minutes, then 200 µl aliquots of lysate from each well were pipetted into a gamma counter tube and counted.

The concentration of ^{125}-I-TGF-β1 were calculated as following; on the vial label the following information was given:

Specific activity (SA) in Ci /m mol =1080

Total activity (TA) in µCi = 10

The contents of the vial was reconstituted in a volume (v) of 100 µl of 0.17 mM HCl. The concentration of TGF-β1 in nM when reconstituted was:

TA/SA x10^{-3}

In this case 10/1080 x10^{-3} nM = 9.26 nM.

10 µCi in 100 µl

= 10^5 µCi in 1 litre

= 0.1 Ci/litre

= 0.1/1080 m mol/liter = 9.26 x 10^{-8}M

Therefore, the stock ^{125}I-TGF-β1 solution was diluted 1 in 9 in binding buffer to produce a solution containing ^{125}I-TGF-β1 at a concentration of approximately 100pM as required for the binding assay.

(*) Binding Buffer-Phenol red-free RPMI+0.1% BSA (no FCS).

2.12 STATISTICS

Student's t test was used for statistical comparisons using a Macintosh computer (Instat program).

CHAPTER III

RESULTS

3.1 CORRELATION OF CELL NUMBER WITH DNA CONTENT

In some experiments with the OAW42 cell line, DNA synthesis was used as a means of assessing cell survival and growth. Therefore, it is important to ensure that the level of DNA will reflect cell number. An initial experiment was carried out to show the correlation between DNA content and cell number.

Methods

The OAW42 cell line was subcultured as in section 2.4.3. of the Methods chapter using 25 cm^2 tissue culture flasks. The cells were then allowed to plate down for 24 hours before the medium was renewed. Every 24 hours, the culture medium was removed from 2 flasks and the cell monolayer washed twice with PBS. The cells were harvested by trypsinisation, a sample of the harvested cells was removed and the number of cells counted using a haemocytometer. The remaining cells were lysed and a DNA assay carried out as described in section 2.5.2. of the Methods chapter.

Results

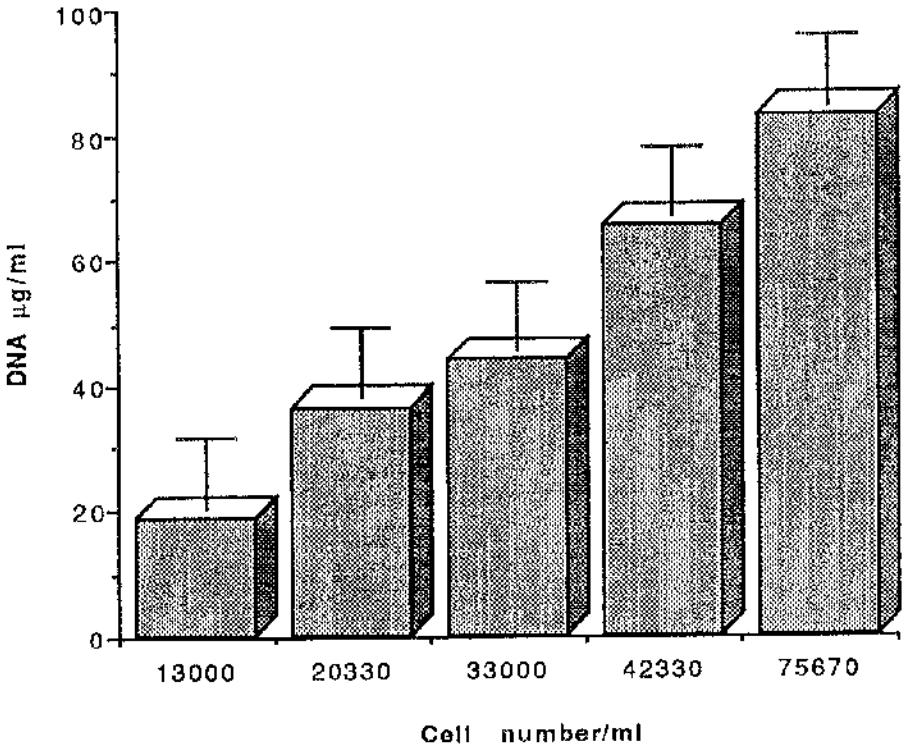
The relevance of using DNA concentration as a measure of cell number or cell proliferation can be validated by the production of a linear correlation curve between the two as seen in (fig.9).

Conclusions

Results in figure (9) show that there is good correlation between the concentration of DNA in a sample as determined by the Hoechst method and the number of cells in the same sample for all incubation times up to 96 hours.

Figure 9. Correlation of DNA concentration with cell number. This graph is an example of correlation between cell number and DNA concentration. Each column represents cell number (for a sample taken at 0, 24, 48, 72 or 96 hours, which was counted using a haemocytometer, the remainder of the same sample being assayed for DNA using the Hoechst Method as described in Methods chapter section 2.5.2.

DNA concentration versus cell number



3.2 CORRELATION OF CELL NUMBER WITH MTT ABSORBANCE

In most experiments with the OAW42 cell line and other cell lines, the MTT cell proliferation assay was used as a means of assessing cell survival and proliferation. Therefore, it was important to ensure that the level of MTT absorbance will reflect cell number.

Method

The OAW42 cell line was subcultured as in section 2.4.3. of the Methods chapter using 96 well tissue culture plates. Cells were plated down into individual plates at 2500 cells/ml, the cells were then allowed to grow for 24 hours before the medium was changed with experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS. One plate was taken after each 24 hours period and 50 µl MTT dye (5 mg/ml in sterilised PBS) was added to each well at half of the plate, the rest of the cells were harvested after 24, 48, and 72 hours. The MTT assay carried out as described in section 2.7.3. of the Methods chapter and total cell number determined for each plate.

Results

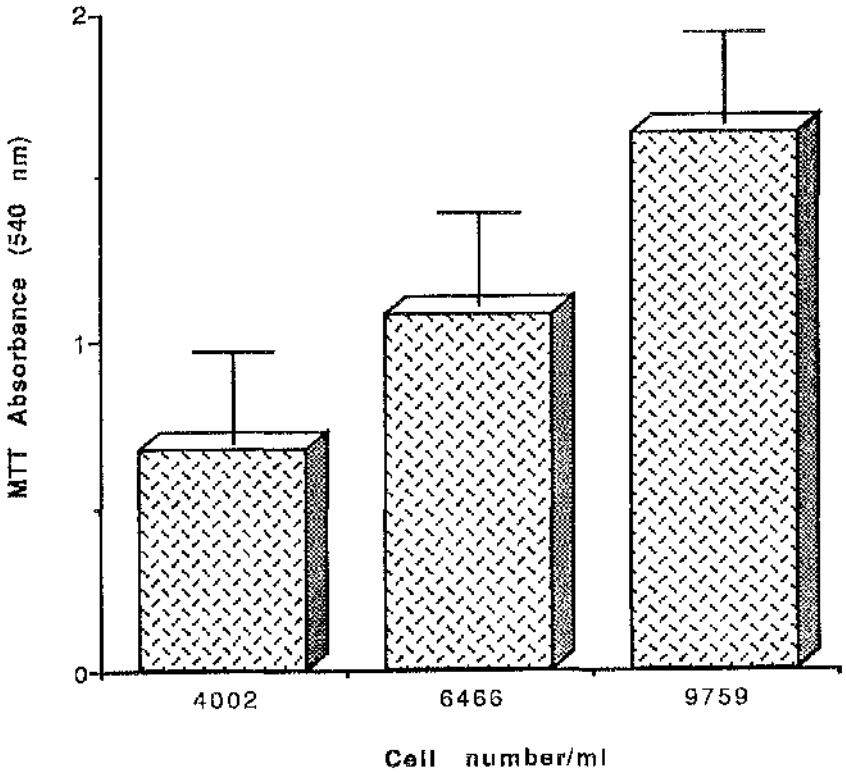
The use of MTT absorbance as a measure of cell number or cell proliferation can be validated if there is a linear correlation when cell number, after different incubation times, is plotted against MTT absorbance. (Fig.10) shows the correlation curve between the two.

Conclusions

Results in (fig.10) show that there was good correlation between the MTT absorbance and the number of cells in the same sample.

Figure 10. Correlation of MTT absorbance with cell number. Each column represents increasing MTT absorbance versus cell number after 24, 48 or 72 hours. The MTT assay was carried out as described in section 2.7.3. of Methods chapter. Values represent mean +/- SEM (bars) of triplicate experiments.

MTT absorbance versus cell number



3.3 GROWTH RESPONSE TO EGF

The effect of various concentration of EGF on growth of ovarian cancer cell lines OAW42, PEO1 and PEO1cddp was studied.

Method

The three cell lines OAW42, PEO1, and PEO1cddp were subcultured as in section 2.4.3. of Methods chapter so that a 20% monolayer was formed within 3x96 well tissue culture plates per cell line as in section 2.7.2. of Methods chapter. The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (Phenol Red Free Medium) supplemented with 5% DHDCCFCS in the presence of increasing concentrations of EGF (from 2.5 to 50 ng/ml) was added to each well. 8 wells were used for each concentration of EGF. Control cells were incubated in experimental medium without additive.

The cells were incubated at 37°C for 72 hours with changes of experimental medium after 48 hours incubation. At each 24 hours time point one plate for each cell line was treated by adding 50 µl MTT dye (concentration 5 mg/ml in sterilised PBS). The MTT assay was carried out as described in section 2.7.3. of the Methods chapter.

Results

The results of the EGF sensitivity studies were expressed in the form of time course and dose response for each of OAW42, PEO1 and PEO1cddp cells.

These are shown as the mean of 3 independent experiments and the standard errors of the mean are indicated by the error bars.

Figure (11) shows the OAW42 cell growth response to EGF. At 24 hours there was decreased cell proliferation at all doses of EGF.

After 48 hours incubation, an increase in cell growth at different doses of EGF was observed, reaching a maximum of about 34% ($p < 0.0001$) above control at 10 ng/ml dose.

At 72 hours exposure, cells showed a dose-dependent increase reaching a maximum of cell proliferation about 21.8% ($p = 0.0005$) above control at 5 ng/ml dose.

Figure 12 shows that PEO1 cell growth responds positively to the influence of EGF. At 24 hours exposure cells showed stimulation of growth reaching a maximum of 18.9% ($p = 0.002$) above control at 2.5 ng/ml dose.

After 48 hours exposure data showed similar results to those at 24 hours reaching a maximum of about 30.8% ($p < 0.0001$) above control at 2.5 ng/ml dose. At 72 hours exposure, EGF still shows positive stimulation to the growth of PEO1 cell line reaching a maximum of 33% ($p = 0.05$) above control at 10 ng/ml dose. EGF inhibit, growth of PEO1 cell line at highest dose (over 50 ng/ml) at all period of incubations.

Figure 13 shows that PEO1cddp cell growth responds differently from the parent cell line PEO1to the influence of EGF. At 24 hours exposure cells demonstrate slight stimulation at a dose of 5 ng/ml about 6.3% ($p = 0.03$) above control. After 48 hours exposure cell growth was inhibited by EGF reaching a maximum of 16.7% ($p = 0.001$) at 50 ng/ml dose. At 72 hours exposure PEO1cddp cell growth was inhibited by EGF at all doses from 2.5 to 50 ng/ml reaching a maximum of about 31.9% ($p < 0.0001$) below control at 20 ng/ml dose.

Conclusion

The effect of EGF on OAW42 cells compared with PEO1 cells as can be seen from both results, the effect of EGF on growth of OAW42 cells shows over 20% stimulation to the growth in between 5 to 10 ng/ml dose after 48 and 72 hours. PEO1 cells shows over 30% stimulation to the growth at doses of 2.5 to 10 ng/ml. These comparison illustrates that both cell lines were growth stimulation to the effect of EGF at different doses.

Figure 11. Growth response of the OAW42 ovarian cancer cell line to Epidermal growth factor (EGF). Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of EGF (2.5 to 50 ng/ml). Values represent mean +/- SEM (bars) of triplicate experiment.

Time course and dose response of OAW42 cells to EGF

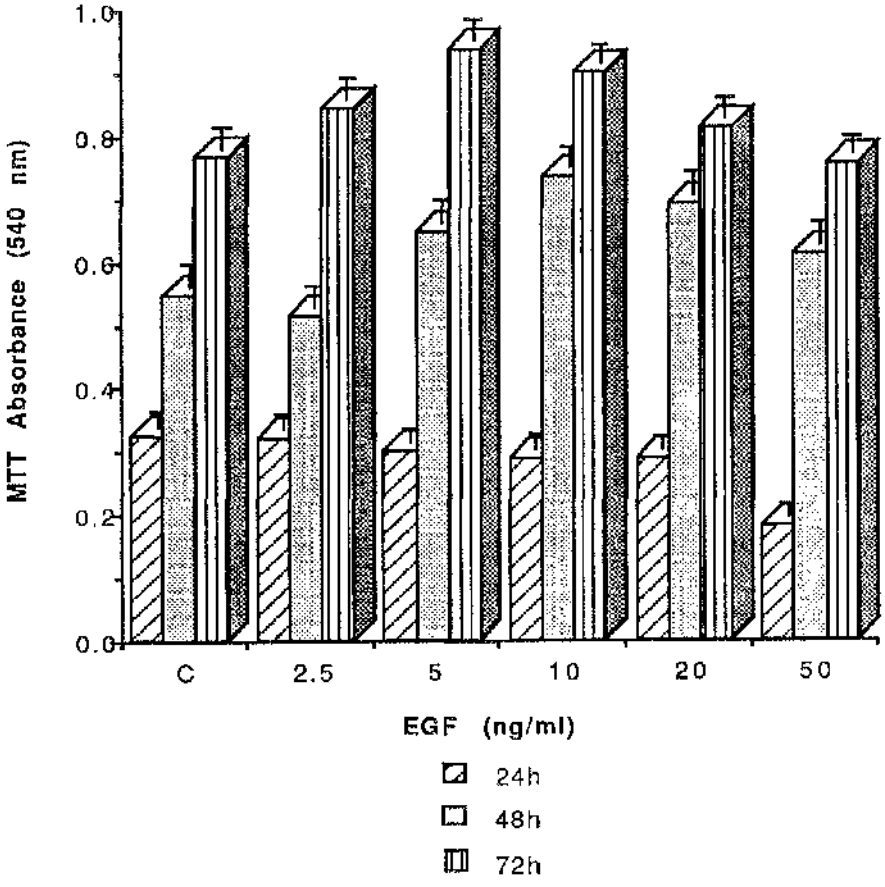


Figure 12. Growth response of human ovarian cancer cells (PEO1) to epidermal growth factor (EGF). PEO1 cells were growing in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of EGF (2.5 to 50 ng/ml). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1 cells to EGF

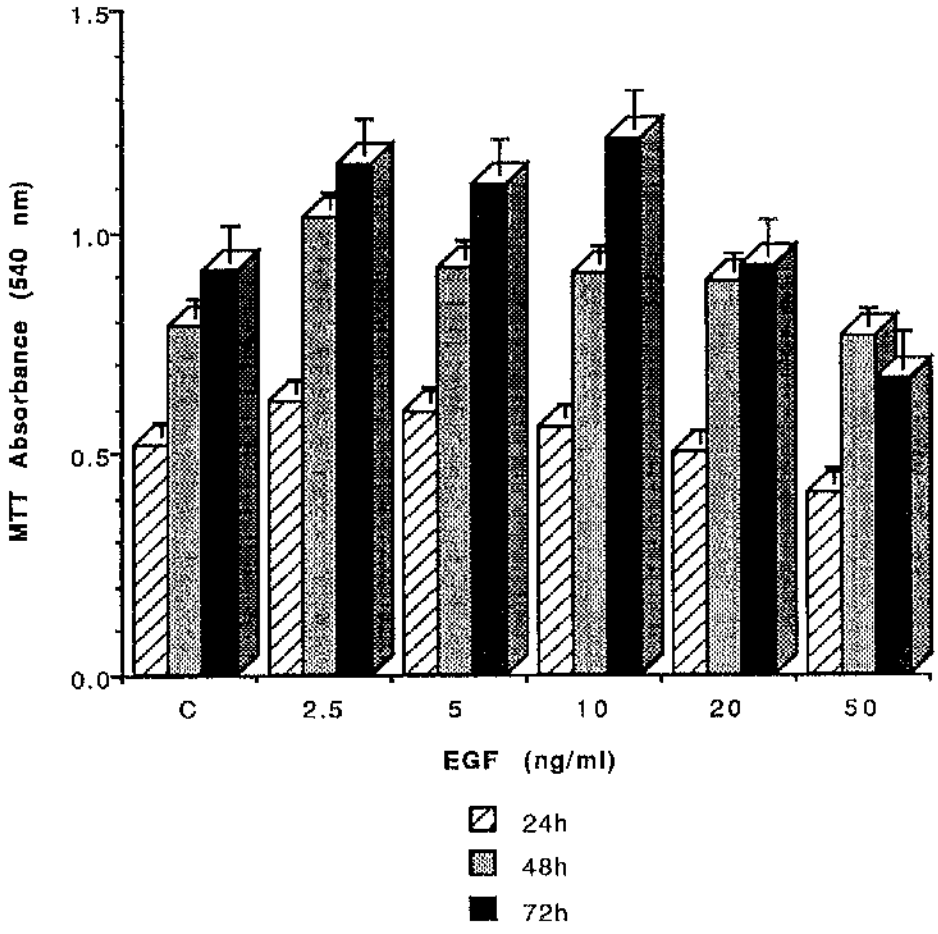
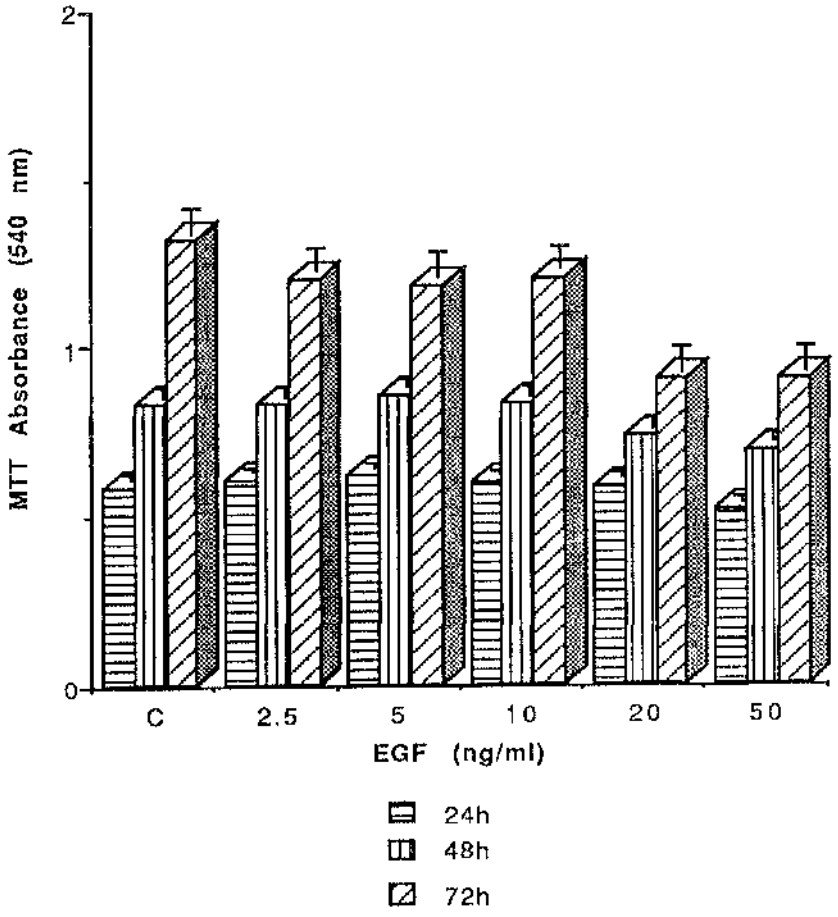


Figure 13. Growth response of the ovarian cancer cell line (PEO1cddp) to epidermal growth factor (EGF). PEO1cddp cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of EGF (2.5 to 50 ng/ml). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1cddp cells to EGF



3.4 GROWTH RESPONSE TO TGFα

These experiments studied the effects of various concentrations of TGF-α on growth of both cell lines OAW42 and PEO1 cells.

Method

The two cell lines were subcultured as in section 2.4.3. of Methods chapter so that a 20% monolayer was formed within 3x96 well tissue culture plates per cell line as in section 2.7.2. of Method chapter. $4x10^3$ cells were added to each well, the cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS in the presence of increasing concentrations of TGF-α (concentration range from 0.5 to 50 ng/ml) was added to each well. 8 wells were used for each concentration of TGF-α. Control cells were incubated in experimental medium without additive. The cells were incubated at 37°C for up to 72 hours with a change of experimental medium after 48 hours. After each 24 hour incubation period one plate for each cell line was treated by adding 50 µl MTT dye (5 mg/ml in sterilised PBS). The MTT assay was carried out as described in section 2.7.3. of the Methods chapter.

Results

The results of the TGF-α sensitivity studies are expressed in the form of time course and dose response histograms for both OAW42 and PEO1 cell lines. These are shown as means of 3 independent experiments and the standard error of the means are indicated by the error bars.

Figure 14 shows that OAW42 cell growth responds positively but in a delayed manner to the influence of TGF-α . At 24 hours there is no growth stimulation of growth. After 48 hours exposure cells showed stimulation to the growth reaching a maximum of 9.3% ($p = 0.0004$) above control at 10 ng/ml and 20% ($p = 0.0002$) above control at 25 ng/ml after 72 hours exposure. The growth stimulation by TGFα was seen in PEO1 cells figure 15 by 24 hours 35% ($p < 0.0001$) above control at 25 ng/ml. After 48 hours exposure, stimulation of the cells were seen in most of TGFα doses reaching a maximum of approximately 41% ($p < 0.0001$) above control at 50 ng/ml TGFα dose. After 72 hours exposure cell proliferation reaching a maximum of approximately 24.4% ($p = 0.0002$) above control at 10 ng/ml dose.

Conclusion

Comment on growth effect of TGFα on OAW42 cells compared with PEO1 cells as can be seen from both results, the effect of TGFα on growth of OAW42 cells shows growth stimulation over 48 hours reaching a maximum of 20% at 25 ng/ml . PEO1 cells shows growth stimulation at 24, 48, and 72 hours with a maximum of 41% at 50 ng/ml dose.

These comparison illustrates that both cell lines were growth stimulation to the different doses of TGFα but PEO1 cells shows more stimulation to influence of TGFα than OAW42 cells.

Figure 14. Growth response of human ovarian cancer cells (OAW42) to transforming growth factor-α (TGF-α). OAW42 cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of TGF-α (0.5 to 50 ng/ml). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of OAW42 cells to TGFα

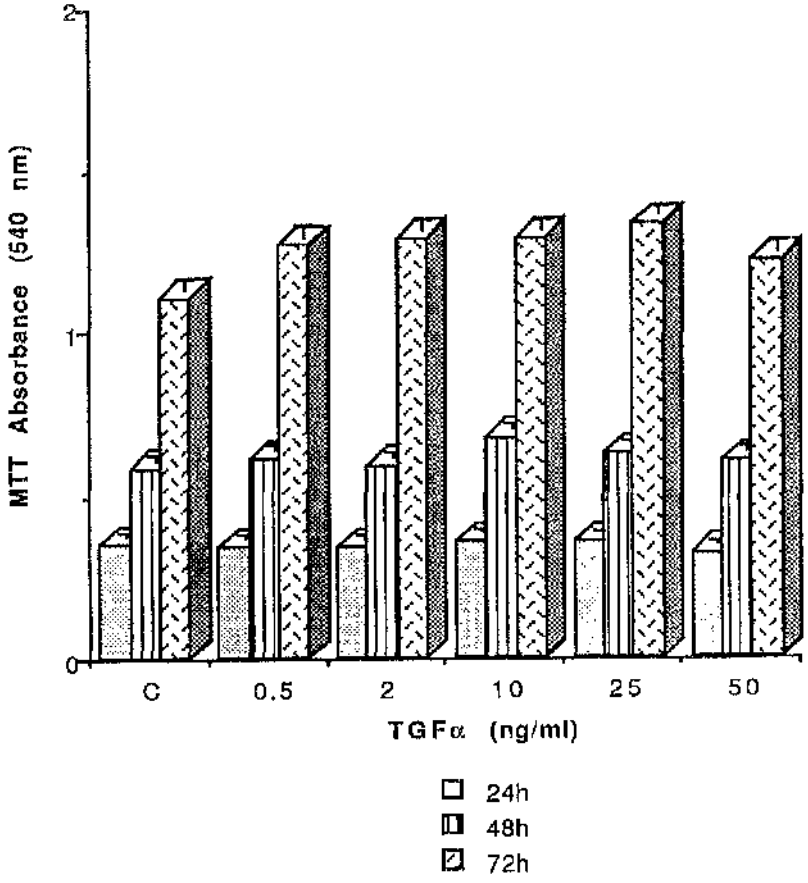
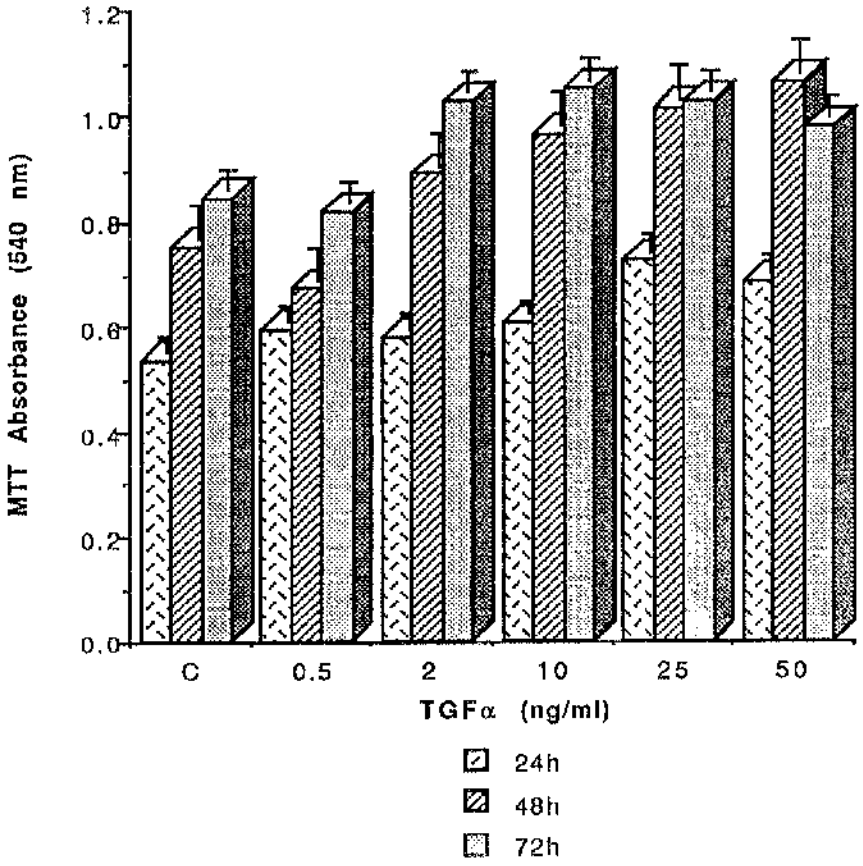


Figure 15. Growth response of human ovarian cancer (PEO1) to transforming growth factor-a (TGF-α). PEO1 cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of TGF-α (0.5 to 50 ng/ml). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1 cells to TGFα



3.5 GROWTH RESPONSE TO TGF-β1

The effects of various concentrations of TGF-β1 on growth of the OAW42 ovarian cancer cell line were studied by measuring cell numbers in terms of DNA content.

Method

OAW42 cells were subcultured using 24 well tissue culture plates.
The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (phenol Red-Free Medium supplemented with 5% DHIDCCFCS) in the presence of increasing concentrations of TGF-β1 concentration range (from 0.05 to 5 ng/ml) was added to each well. 4 wells were used for each concentration of TGF-β1. Control cells were incubated in experimental medium without additive. The cells were incubated at 37°C for 96 hours with changes of experimental medium each 48 hours. The culture medium was removed and the cell monolayer were washed twice with PBS. The cells were harvested by trypsinisation and lysed and DNA assay carried out as described in section 2.5.2. of the Methods chapter.

Results

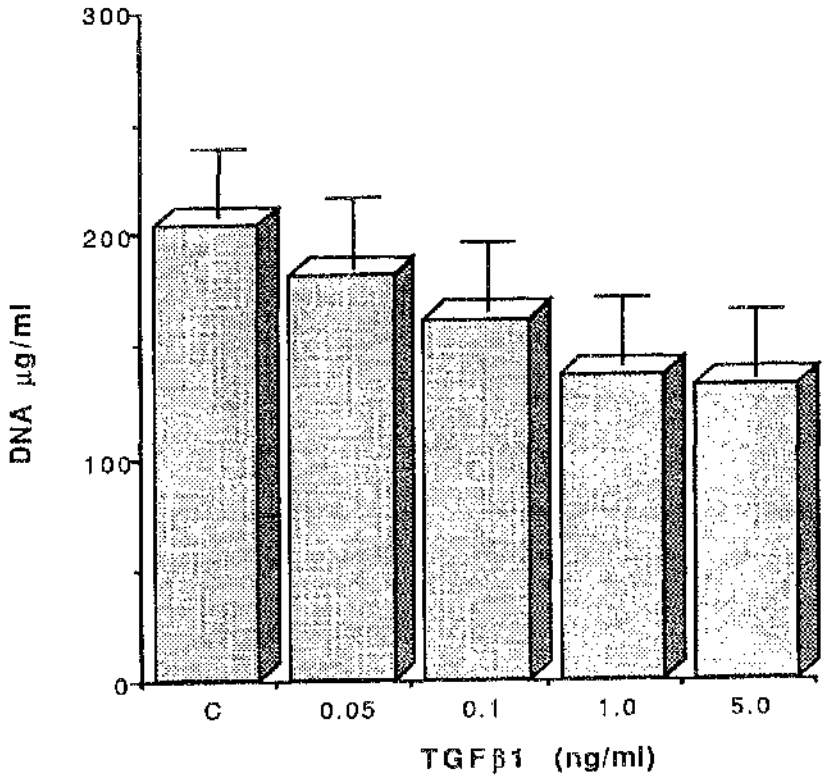
The results of the TGF-β1 sensitivity studies are expressed in the form of a dose response curve. Figure 16 illustrates the dose response of the OAW42 cell line. These are shown as means of 3 independent experiments and the standard error of the mean is indicated by the error bars.

Conclusions

OAW42 cells are sensitive to increasing concentrations (from 0.05 to 5 ng/ml) of TGFβ1 over 96 hours exposure. There was a significant decrease of DNA content (33.5% and 35.9% at 1.0 and 5.0 ng/ml, respectively) $p < 0.05$ after 96 hours incubation period compared with control cells over the same time .

Figure 16. Growth response of OAW42 ovarian cancer cells to transforming growth factor-β1 (TGF-β1). Cells were growing in experimental medium (Phenol Red Free Medium) supplemented with 5% and exposed for 96 hours to increasing concentrations of TGF-β1 (0.05 to 5 ng/ml).Values represent mean +/- SEM (bars) of triplicate experiments.

Dose response of OAW42 cells to TGFβ1



3.6 GROWTH RESPONSE TO LH

The effects of various concentration of Luteinizing hormone (LH) on growth of both cell lines OAW42 and PEO1 cells were studied.

Method

The two cell lines were subcultured as in section 2.4.3. of the Methods chapter so that a 20% monolayer was formed within 3x96 well tissue culture plates per cell line as in section 2.7.2. of the Methods chapter. The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS in the presence of increasing concentration of LH, concentration range (from 2.0 to 60 iu/l) was added to each well. 8 wells were used for each concentration of LH. Control cells were incubated in experimental medium without additive. The cells were incubated at 37°C for a maximum of 72 hours with changes of experimental medium each 48 hours. After each 24 hours incubation, one plate for each cell line was treated by adding 50 µl MTT dye concentration (5 mg/ml in sterilised PBS). The MTT assay carried out as described in section 2.7.3. of the Methods chapter.

Results

The results of the LH sensitivity studies are in the form of time course and dose response curves for the OAW42 and PEO1 cell lines. These results are shown as the mean of 3 independent experiments and the standard errors of the mean are indicated by the error bars.

Conclusions

The growth of OAW42 cells and PEO1 cell is inhibited by increasing concentrations of LH (from 2 to 60 iu/l) over 72 hours exposure.

Figure 17 shows the data for OAW42 cells.

At 24 hours the data showed a slight rise in cell proliferation at a dose of 10 iu/l and inhibition of cell growth of 21.3% ($p = 0.008$) below control level at 60 iu/l dose.

After 48 hours exposure, OAW42 cells showed a greater inhibition of growth reaching a maximum of 32% ($p < 0.0001$) below control levels at 60 iu/l dose.

After 72 hours exposure OAW42 cells showed the same result as at 48 hours of time course.

Figure 18 shows the data for PEO1 cells. At 24 hours the data showed a dose-dependent inhibition reaching a maximum at 60 iu/l dose of 34.2% ($p = 0.0005$) below control cell growth.

PEO1 cells after 48 hours exposure showed similar result as at 24 hours exposure with increasing inhibition of growth, reaching a maximum of 26.9% ($p = 0.001$) below of control level at 60 iu/l dose.

After 72 hours exposure PEO1 cells showed slight stimulation of growth at lower dose of LH and highest inhibition of 32% ($p < 0.0001$) below control level at 60 iu/l dose.

Figure 17. Growth response of human ovarian cancer cells OAW42 to Luteinizing hormone (LH). OAW42 cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of LH (2 to 60 iu/l). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of OAW42 cells to LH

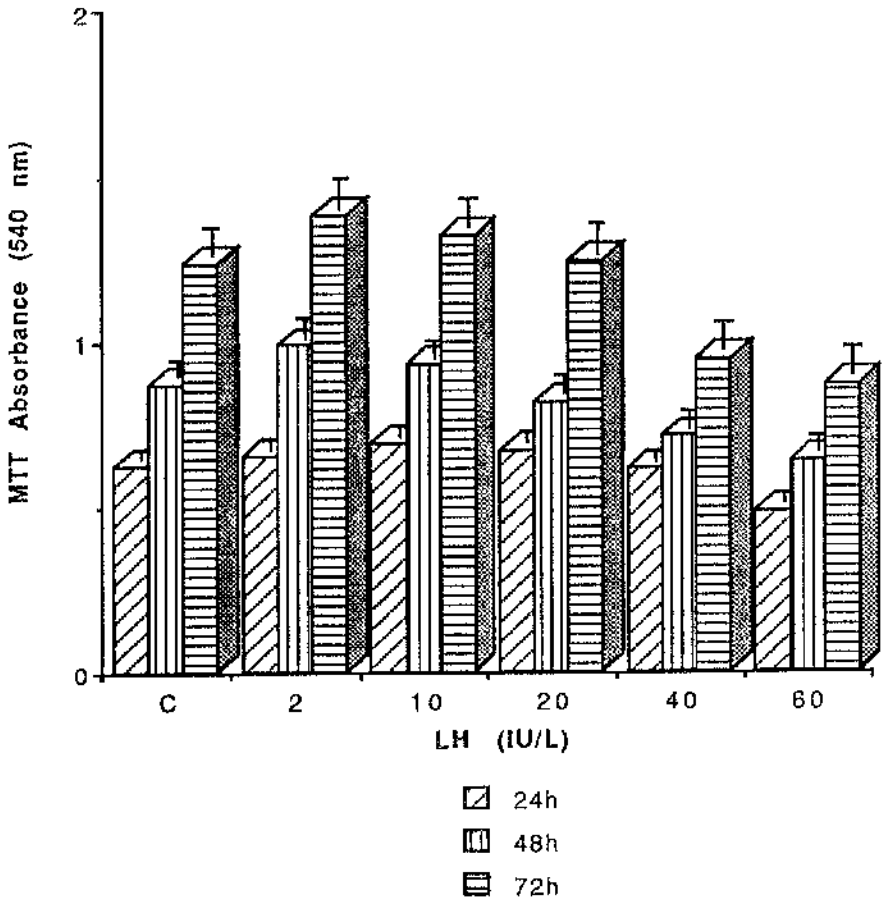
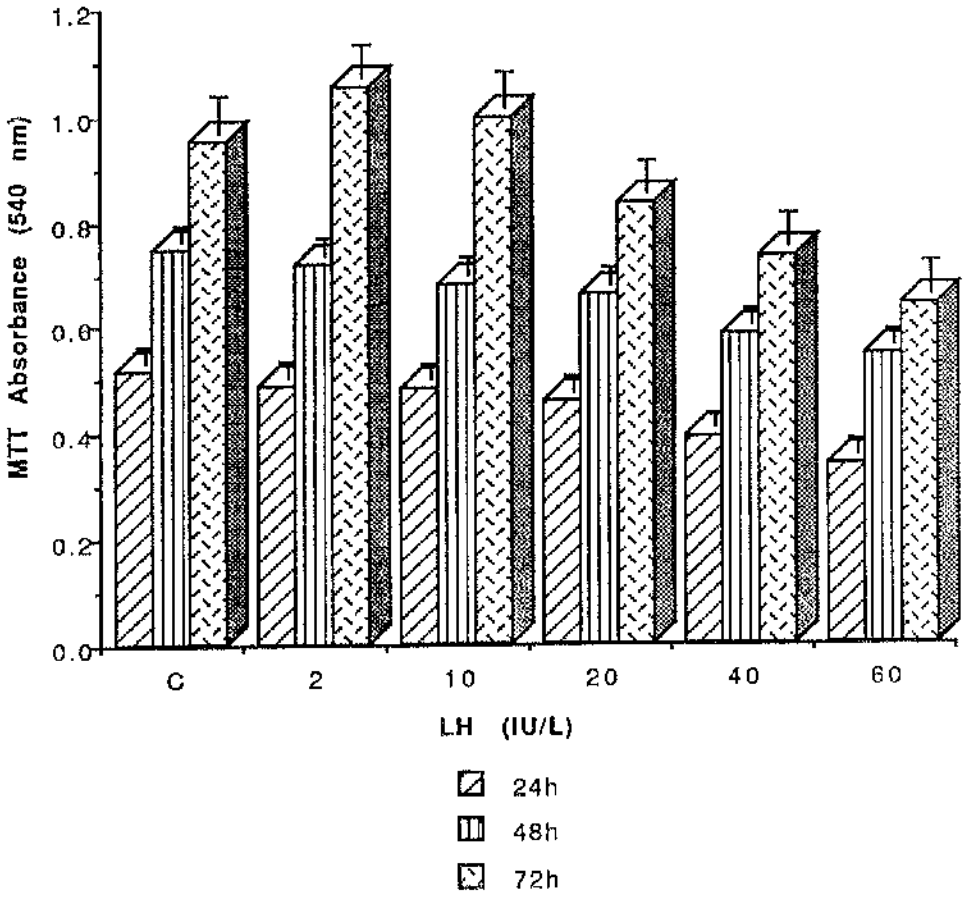


Figure 18. Growth response of human ovarian cancer cells PEO1 to Luteinizing hormone (LH). PEO1 cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of LH (2 to 60 iu/l). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1 cells to LH



3.7 STUDIES WITH ANTI-TGF-β1 ANTIBODY

Growth effects of LH and anti-TGF-β1 antibody in OAW42 cells.

Method

OAW42 cells were subcultured as in section 2.4.3. of the Methods section. Cells ($2x10^4$ cells/well) were added to each well of a 24 well tissue culture plates. The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS in presence of LH (40 iu/l) or LH + TGF-β1-antibody (5 or 30 µl) was added to each well. 2 wells were used for each condition. Control cells were incubated in experimental medium without additive. The cells were incubated at 37°C for 48 hours. The culture medium was removed and the cell monolayer were washed twice with PBS. The cells were harvested by trypsinization, lysed and DNA assay carried out as described in section 2.5.2. of the Methods chapter.

Results

The results of incubation of OAW42 cells in experimental medium containing LH +/- anti-TGF-β1 antibody are expressed relative to those in control medium.

These results are shown as the mean of 2 independent experiments and the standard errors of the mean are indicated by the error bars Figure 19.

Conclusions

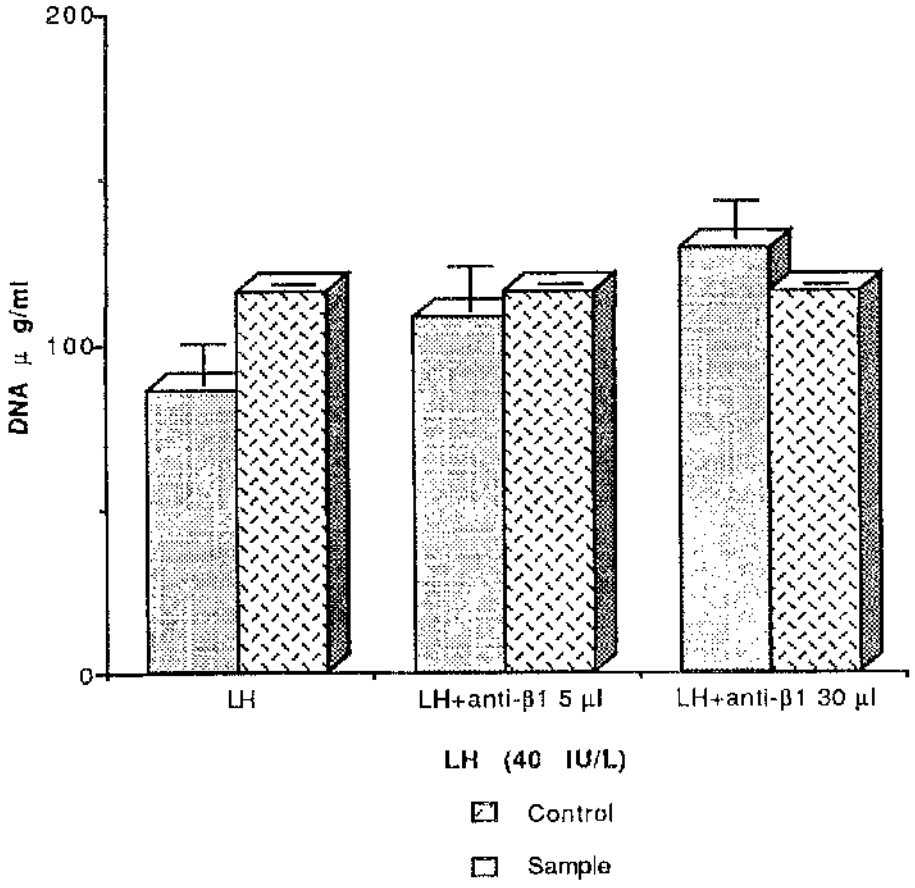
Previous studies had shown that the maximum inhibition of the growth of OAW42 cells was at a dose of 40 iu/l LH.

Coincubation of LH (40 iu/l) with the anti-TGF-β1 antibody at 5 µl/ml essentially blocked the anti-proliferative effect of LH alone. A Further increase in the concentration of antibody up to 30 µl/ml completely reversed the cytostatic effect of

LH and induced growth above the level of control, suggesting that under control conditions, growth may be partially blocked by some endogenous TGFβ-like activity. LH alone significantly inhibits growth of OAW42 cells. In the presence of 5 μg/ml anti-TGFβ antibody, this growth inhibition appears to be reversed although there is no significant difference between either LH alone and LH + 5 μg/ml anti-TGFβ antibody and control. Increasing the concentration of antibody present in the culture medium, caused a stimulatory effect. Thus, 30 μl/ml of antibody was enough to overcome the inhibitory action of LH and, indeed allowed growth above control, suggesting that TGF-β1 acts as an autocrine growth inhibitory factor in this cell line.

Figure 19. Growth effects of LH and anti-TGFβ1 antibody in OAW42 cells. Cells were grown in experimental medium supplemented with 5% DHIDCCFCS and exposed for 48 hours to LH (40 iu/l) +/- anti-TGFβ1 antibody (5 or 30 μl/ml). Values represent mean +/-SEM (bars) of duplicate experiments.

Studies with anti-TGFβ1 antibody



3.8 THE INTERACTION BETWEEN EGF, LH AND FSH

The ability of EGF to modulate the growth regulation of OAW42 cells by the gonadotrophins LH and FSH was studied over 96 hours.

Method

OAW42 cells were subcultured using 24 well tissue culture plates, $2x10^4$ cells/well. The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS in the presence of various combinations of EGF (10 ng/ml), LH (40 iu/l) and FSH (30 iu/l). Control cells were incubated in experimental medium without additives.

The cells were incubated at 37°C for 96 hours with changes of medium and drugs each 48 hours. The culture medium was removed and the cell monolayer were washed twice with PBS. Cells were harvested by trypsinization and lysed and the DNA assay carried out as described in section 2.5.2. of Methods chapter.

Results

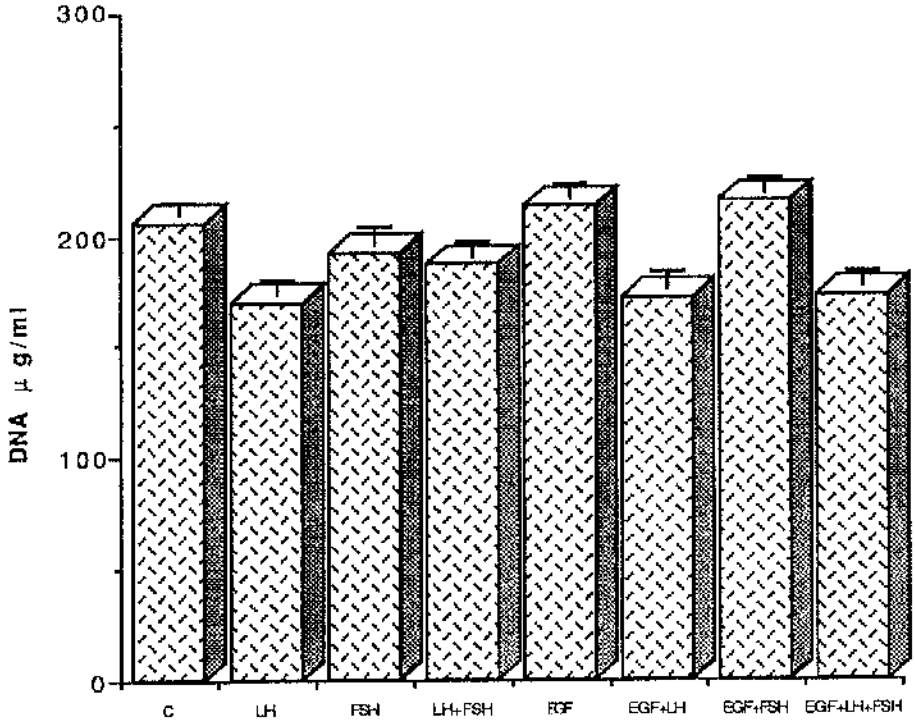
The interactions between EGF, LH, and FSH are shown as means of 3 independent experiments in figure (20) and the standard errors of the mean are indicated by the error bars.

Conclusions

As can be seen from figure 20, LH alone caused the expected growth inhibition, whereas FSH alone caused no significant change in growth over 96 hours. EGF alone induced a small growth stimulation about 5% ($p = 0.02$) that was reversed by LH (and by LH + FSH) but not by FSH alone.

Figure 20. The effect of EGF with both gonadotrophin hormone LH and FSH on growth of OAW42 human ovarian cancer cell line. Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 96 hours to (EGF 10 ng/ml, LH 40 iu/l , and FSH 30 iu/l). Values represent mean +/- SEM (bars) of triplicate experiments.

Dose response of OAW42 cells to EGF, LH and FSH



EGF 10 ng/ml

LH 40 IU/L

FSH 30 IU/L

3.9 GROWTH RESPONSE TO OESTRADIOL AND PROGESTERONE

The mature ovary actively synthesizes and secretes a variety of hormones. Among these are the sex steroids, which include oestrogens and, during the luteal phase of the cycle, progesterone. It was of interest to establish whether the three ovarian cancer cell lines (OAW42, PEO1, and PEO1cddp) were themselves sensitive to oestrogen and progesterone.

Method

The three cell lines were sub cultured as in section 2.4.3. of the Methods chapter by adding sufficient cells $4x10^3$ cells/well to form a 20% monolayer within 3x96 well tissue culture plates per cell line. The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium containing different concentrations of E_2, and Pg (concentration range from $1x10^{-11}$M to $1x10^{-5}$M) was added to each well. The E_2 and Pg were dissolved in absolute alcohol to form a stock solution of $1x10^{-3}$M which could then be diluted into culture medium to produce working concentrations of 10^{-5} M. 8 wells were used for each dose concentrations and control. Control cells were incubated in experimental medium without hormone additive but with the same final absolute alcohol concentration as the experimental medium. The cells were incubated at 37°C for 72 hours with changes of experimental medium after 48 hours incubation.

After every 24 hours exposure, one plate for each cell line was treated by adding 50 µl MTT dye (concentration 5 mg/ml in sterilised PBS). The MTT assay was carried out as described in section 2.7.3. of the Methods chapter.

Results

The results of the E_2 and Pg sensitivity studies are expressed in the form of a time course and dose response for OAW42, PEO1 and PEO1cddp cells.

These are shown as mean of 3 independent experiments and the standard errors of the mean are indicated by the error bars.

Figure 21 shows growth of the OAW42 cells is inhibited by increasing concentrations of E2 (from 10^{-11} to 10^{-5}M) over 72 hours exposure. At 24 hours data showed a significant, dose-related inhibition of growth reaching a maximum effect at 10^{-6} M E_2 of 53.4% ($p < 0.0001$) with respect to control. After 48 hours, exposure cells demonstrated similar results to 24 hours, reaching a maximum of 52.9% ($p < 0.0001$) inhibition at 10^{-7} M dose. At 72 hours exposure, data showed highly significant inhibition of cell proliferation at 10^{-6}M E2 giving growth of 59% ($p = 0.0006$) below control.

Figure 22 shows growth of the PEO1 cells is inhibited by different doses of E_2 at all periods of incubation with significant decrease of the cell inhibition reaching a maximum of 32.3% ($p < 0.0001$) below control at 10-6 dose, 45.5% ($p < 0.0001$) at 10^{-7}M dose and 29.8% ($p < 0.0001$) below control at 10^{-6}M dose after 24, 48 and 72 hours exposure respectively.

PEO1cddp in figure 23 shows similar results to those for OAW42 and PEO1 cells with an inhibition of proliferation at 10^{-6}M of 29.1% ($p = 0.003$) below control at 48 hours exposure and 25.3% ($p = 0.006$) after 72 hours exposure at 10^{-7}M.

Conclusion

E2 has a more dramatic inhibition of the growth of OAW42 cells compared with PEO1 cells. The effect of E2 on growth of OAW42 cells shows over 50% inhibition to the growth in between 10^{-7} to 10^{-6}M after 24, 48, and 72 hours. PEO1 cells shows growth inhibition over 25% at 10^{-6}M dose of E2 after 24, 48, and 72 hours.

Progesterone

The results of the Pg sensitivity studies are expressed in the form of time course and dose response to OAW42, PEO1, and PEO1cddp cells. These are shown as mean of 3 independent experiments and the standard deviation is indicated by the error bars.

Results

Figure 24 shows the growth of OAW42 cells is inhibited by increasing concentrations of Pg (from 10^{-12} to 10^{-6}M) over 72 hours exposure. At 24 hours data showed inhibition of growth reaching a maximum effect of 40.4% ($p = 0.0001$) below control cell growth at 10^{-6}M dose. After 48 hours exposure to Pg, inhibition of cell growth was noted for doses 10^{-10} M and above, with a maximum inhibition of 38.2% ($p = 0.3$) at 10^{-6}M dose. After 72 hours exposure, the results showed inhibition of growth reaching a maximum of 42% ($p = 0.009$) at 10^{-6}M dose. After 24 hours exposure PEO1 cells were inhibited at all doses of Pg, as can seen from figure 25, reaching a maximum of 40.2% ($p = 0.004$) at 10^{-6}M dose. After 48 hours incubation, cells were growth inhibited reaching a maximum of 34.1% ($p = 0.003$) below control at 10^{-6}M dose. After 72 hours exposure, inhibition of cell growth was seen at all doses of Pg, reaching a maximum of 44.4% ($p < 0.0001$) at 10^{-6}M dose.

Figure 26 shows that PEO1cddp cell growth was only inhibited by Pg at 10^{-8} and 10^{-6}M dose, reaching a maximum of 26.6% ($p = 0.003$) and 34.8% ($P = 0.002$) below control cell growth after 24 hours. After 48 hours exposure, inhibition of cell growth was seen for all doses reaching a maximum of 24.5% ($p = 0.005$) at 10^{-6}M dose. After 72 hours incubation the growth of PEO1cddp cells was inhibited at all doses, reaching a maximum of 30.2% ($p = 0.0001$) below control cell growth at 10^{-6}M dose of Pg.

Conclusion

The effect of Pg on the growth of OAW42 cells was compared with PEO1 and PEO1cddp cells as can be seen from the results, the effect of Pg on growth of OAW42 cells shows over 40% inhibition to the growth at 10^{-6}M after 24, 48, and 72 hours. PEO1 cells shows growth inhibition from 34 to 44% at 10^{-6}M after 24, 48, and 72 hours. PEO1cddp cells shows growth inhibition over 30% at 10^{-6}M after 24, 48, and 72 hours. these comparison illustrates that Pg more effected on growth of OAW42 and PEO1 cells than PEO1cddp cell line.

Figure 21. Dose response of OAW42 human ovarian cancer cells to oestradiol. OAW42 cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations (10^{-11} to 10^{-5}M) of 17β-oestradiol (E_2). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of OAW42 cells to oestrogen

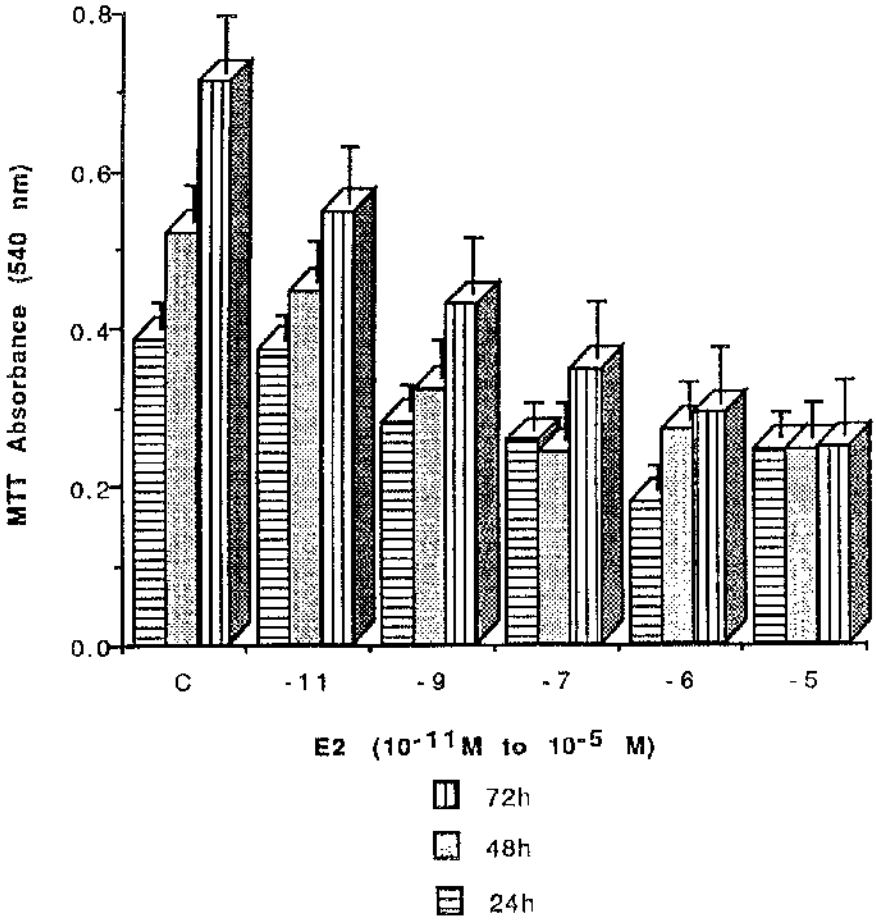


Figure 22. Dose response of PEO1 human ovarian cancer cells to oestradiol. PEO1 cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations (10^{-11} to 10^{-5}M) of 17β-oestradiol (E_2). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1 cells to oestrogen

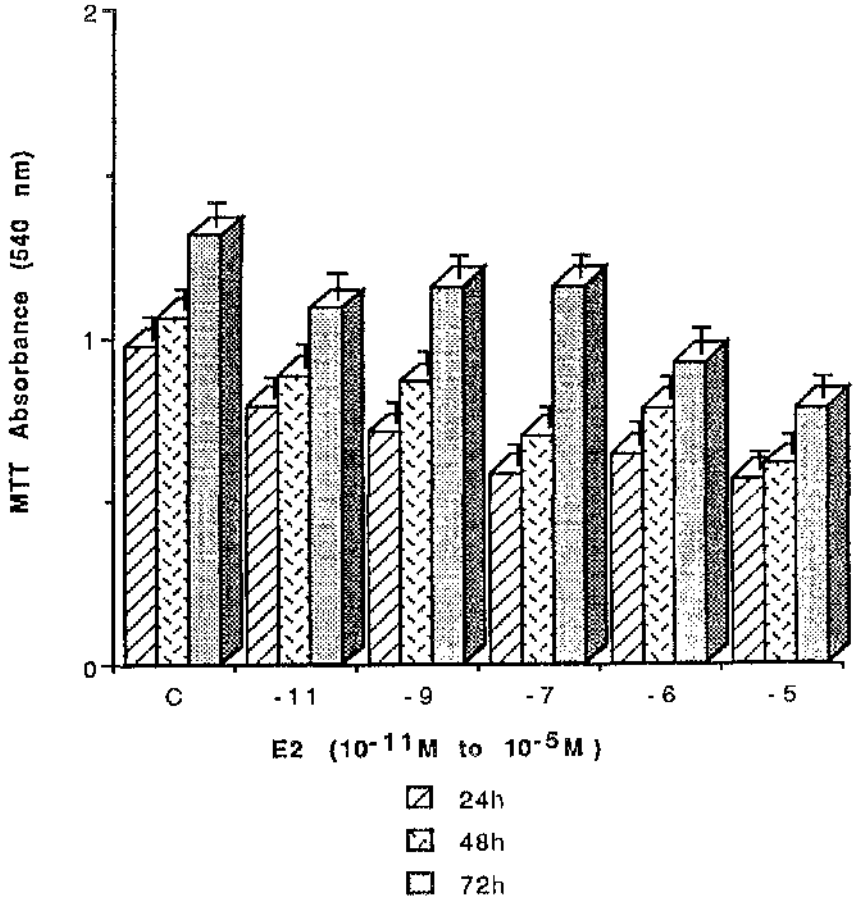


Figure 23. Dose response of PEO1cddp human ovarian cancer cells to oestradiol. PEO1cddp cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations (10^{-11} to 10^{-5}M) of 17β-oestradiol (E_2). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1cddp cells to oestrogen

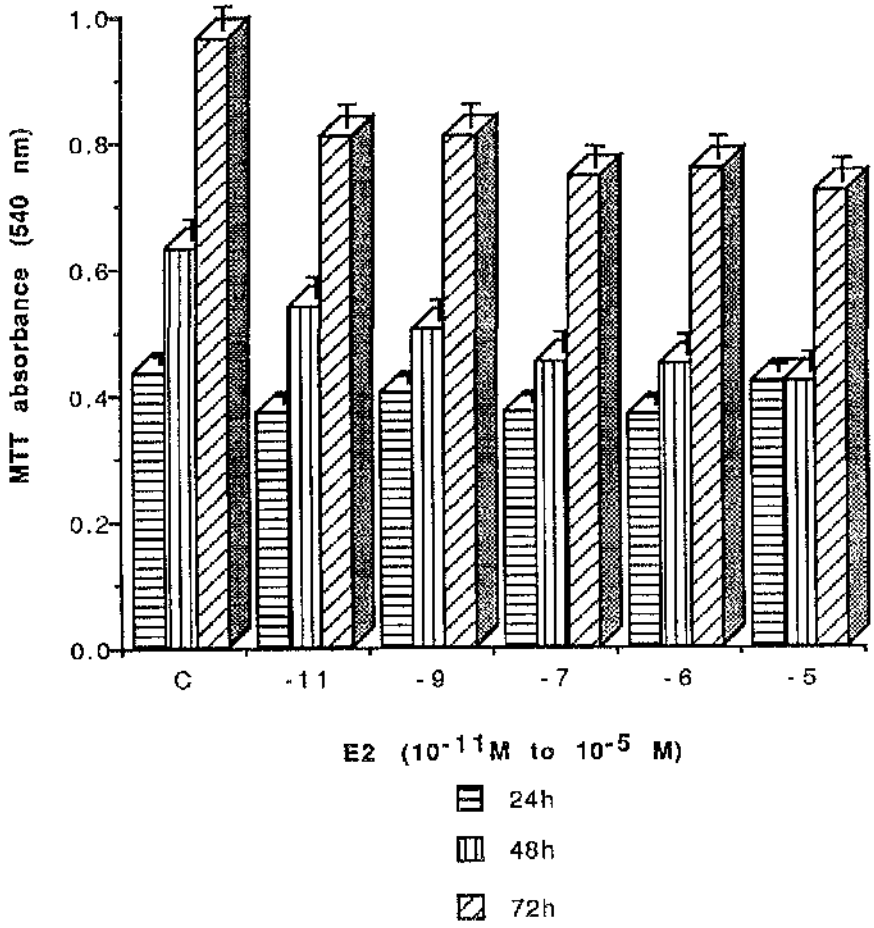


Figure 24. Dose response of human ovarian cancer cells to Pg. OAW42 cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations (10^{-11} to 10^{-6}M) of progesterone (Pg)). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of OAW42 cells to progesterone

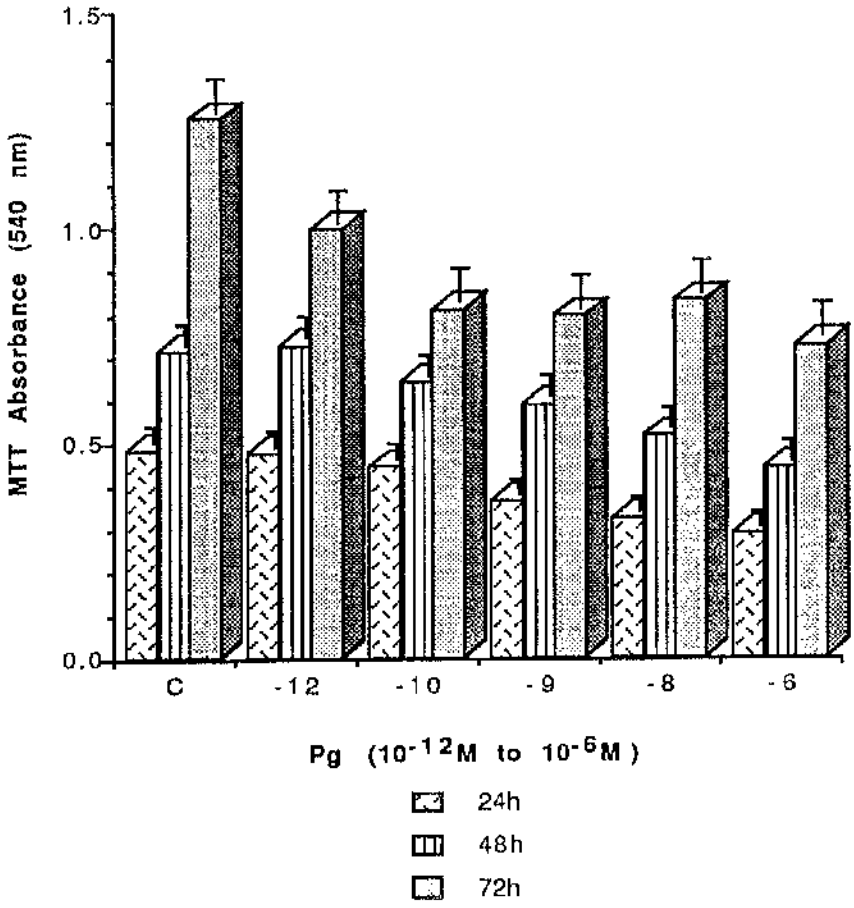


Figure 25. Dose response of human ovarian cancer cells to Pg. PEO1 cells growing in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations (10^{-11} to 10^{-6}M) of progesterone (Pg). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1 cells to progesterone

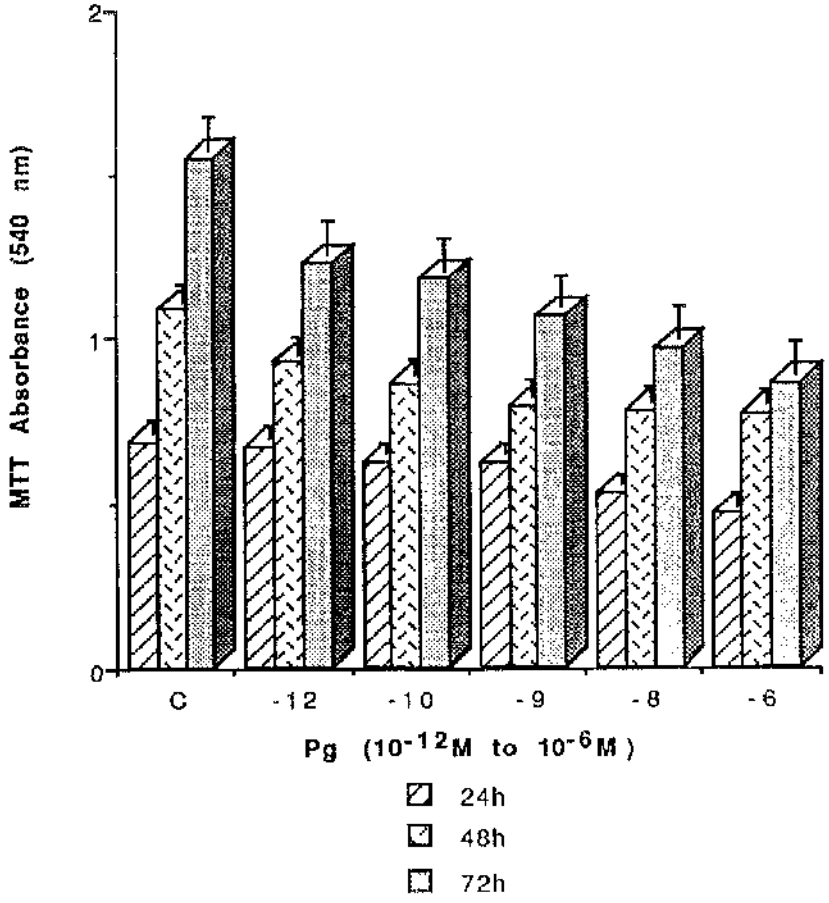
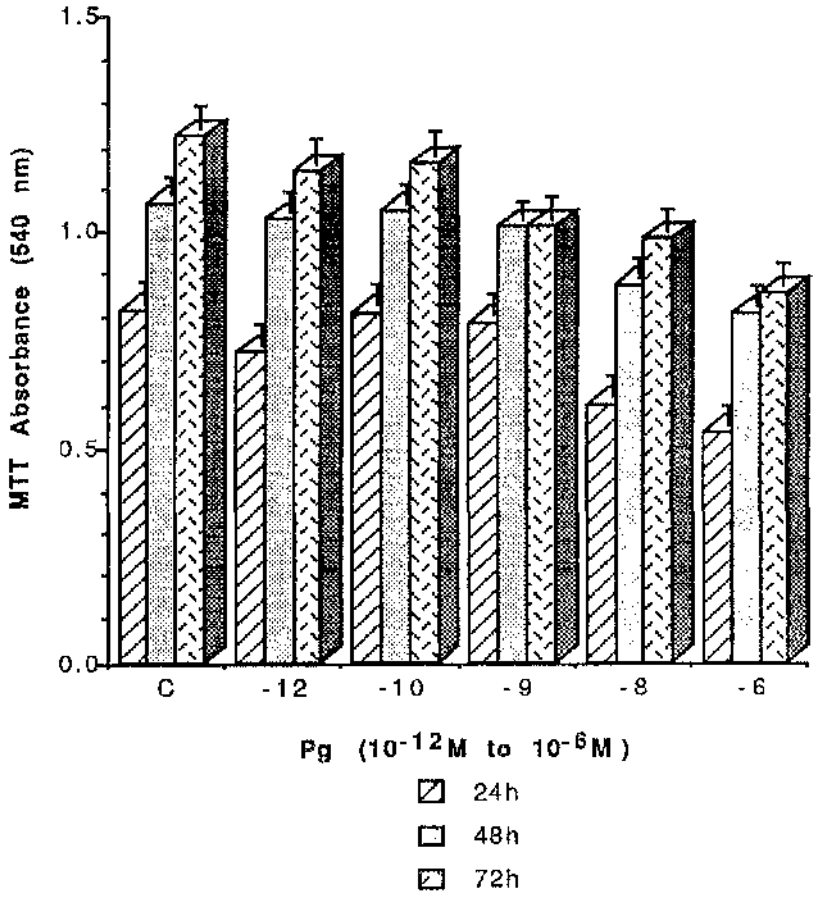


Figure 26. Dose response of human ovarian cancer cells to Pg. PEO1cddp cells were growing in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations (10^{-11} to 10^{-6}M) of progesterone (Pg). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1cddp cells to progesterone



3.10 EPIDERMAL GROWTH FACTOR RECEPTOR

The EGFr is the common cell surface receptor for a family of closely related growth factors (EGF-like factors) including epidermal growth factor (EGF), and transforming growth factor alpha (TGFα). EGFr has been noticed in malignancies of various organs. In ovarian carcinomas EGFr, has been said to be associated with tumour sensitivity to cisplatin (Bauknecht *et al* 1990). A positive EGFr is associated with increased remission rates after cis-platin therapy.

Method

The Vienna lab EGFr 'Scatchard assay' kit (see Methods chapter 2.8) provides materials for the quantitative measurement of epidermal growth factor receptor (EGFr).

Radio receptor assay of EGFr in ovarian tumour cell lines was carried out through incubation of cell membrane suspension (prepared as described in Methods 2.8.7). Membrane suspension (75µl) was incubated with 75µl ^{125}I-EGF in the presence or absence of 1 nM unlabelled EGF for 2 hours at room temperature following the procedure illustrated in section 2.10. of the Methods chapter.

Results

Results of Scatchard analysis of EGFr content of the two cell lines are reported in Table (3).

Table (3). Epidermal growth factor receptor levels in OAW42 and PEO1 human ovarian cancer cell lines.

Cells	Kd (nM)	Fmol/mg protein
OAW42	0.833 +/- 0.019	24.8 +/-5.9
PEO1	0.840 +/-0.023	21.5 +/-1.7

Values represent mean +/- SD triplicate experiments.

Kd = dissociation constant.

Conclusion

OAW42 and PEO1 cells exhibited high affinity EGF receptor with a maximum binding capacity of 24.8 and 21.5 fmol/mg protein and dissociation constant, 0.833 and 0.840 nM respectively as can seen in Table (3). Scatchard plots of EGFr in both cell lines are illustrated in (Fig. 27, 28). Comparing these results with Owens results for clinical tissue, it follows that the EGFr in the two cell lines is very similar to that detected in EGFr positive ovarian cancer biopsies. EGF was present in 39.7% of samples of ovarian tumours with high affinity (Kd < 1 nM) and low affinity (Kd > 1 nM) Owens *et al* (1991). The two cell lines are very similar in EGFr concentration comparing to the PEO1 value, 22 fmol/mg protein (Kd 2.8 nM) Crew *et al* (1992).

Figure 27. Radioreceptor assay of epidermal growth factor receptor (EGFr) in human ovarian cancer cells. Scatchard plot is shown of specific high affinity EGF binding in OAW42 cells. Each data point was performed in triplicate experiments. Both dissociation constant (Kd) and concentration values are reported in Table 3.

EGFR Scatchard Plot in OAW42

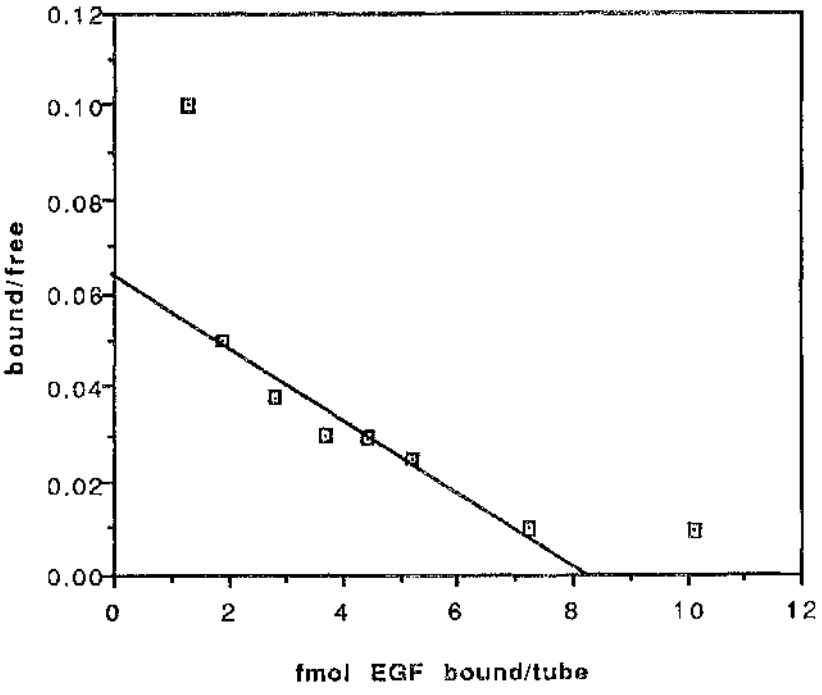
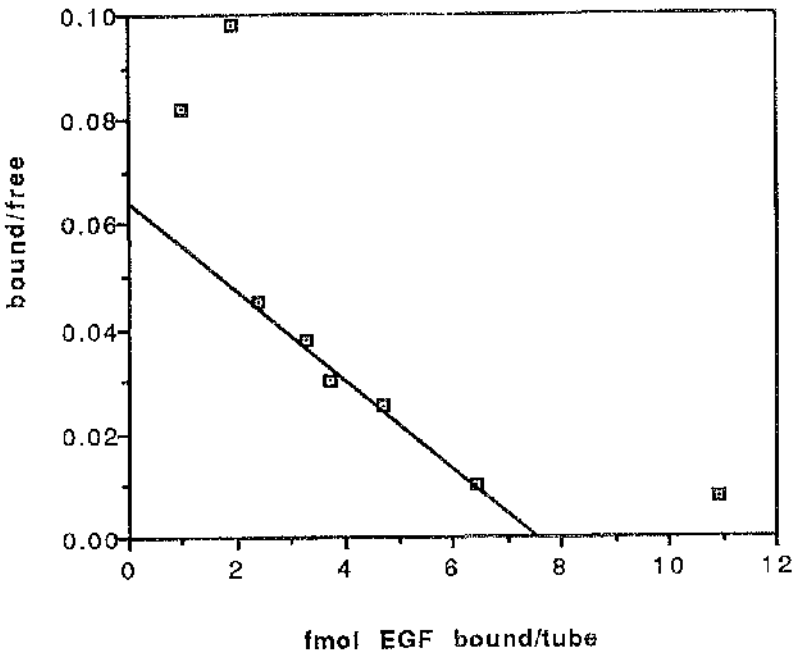


Figure 28. Radioreceptor assay of epidermal growth factor receptor (EGFr) in human ovarian cancer cells. Scatchard plots of specific high affinity EGF binding in PEO1 cells. Each data point was performed in triplicate experiments. Both dissociation constant (Kd) and concentration values are reported in Table 3.

EGFR Scatchard Plot in PEO1 Cells



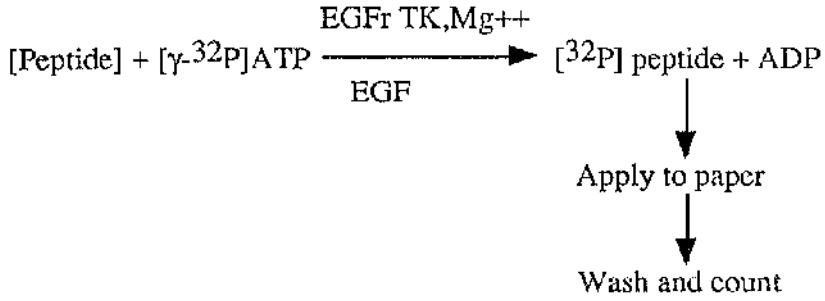
3.11 EGF-RECPTOR TYROSINE KINASE STUDIES

The epidermal growth factor receptor (EGFr) has been recognised as a marker of poor prognosis in some gynecologic cancers. Amersham have developed a reagent which is said to measure the tyrosine kinase activity of the solublised receptor. This kit was used to detect functional EGF receptor in OAW42 ovarian cancer cells on the assumption that only EGF receptor possessing TK activity can be functional.

Method

OAW42 cells were subcultured as in section 2.4.3. of the Methods chapter. Cells were washed, lysed and fractionated as in section 2.8.3. Methods chapter. Enzyme reactions were done in a total volume of 40 µl containing 50 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 2 mM EDTA, 20 µg/ml soybean trypsin inhibitor, 50 µg/ml PMSF, 5 µl of ATP containing 0.25 µCi of [^{32}P] ATP, varying concentrations of Zeneca inhibitor (ZM 252868), and 10 µl of substrate buffer peptide. The reaction was initiated by addition of the labelled ATP. After 30 minutes incubation at 30°C the reaction was terminated by addition of 10 µl stop reagent (solution of 300 mM orthophosphoric acid containing carmosine red) and the solution was passed through a 2.5 cm phosphocellulose filter disk that bound the peptide. The filter was washed five times with 1% acetic acid and distilled water. The filters were placed in vials with 5 ml of scintillation fluid and counted.

Summary of the assay



Results

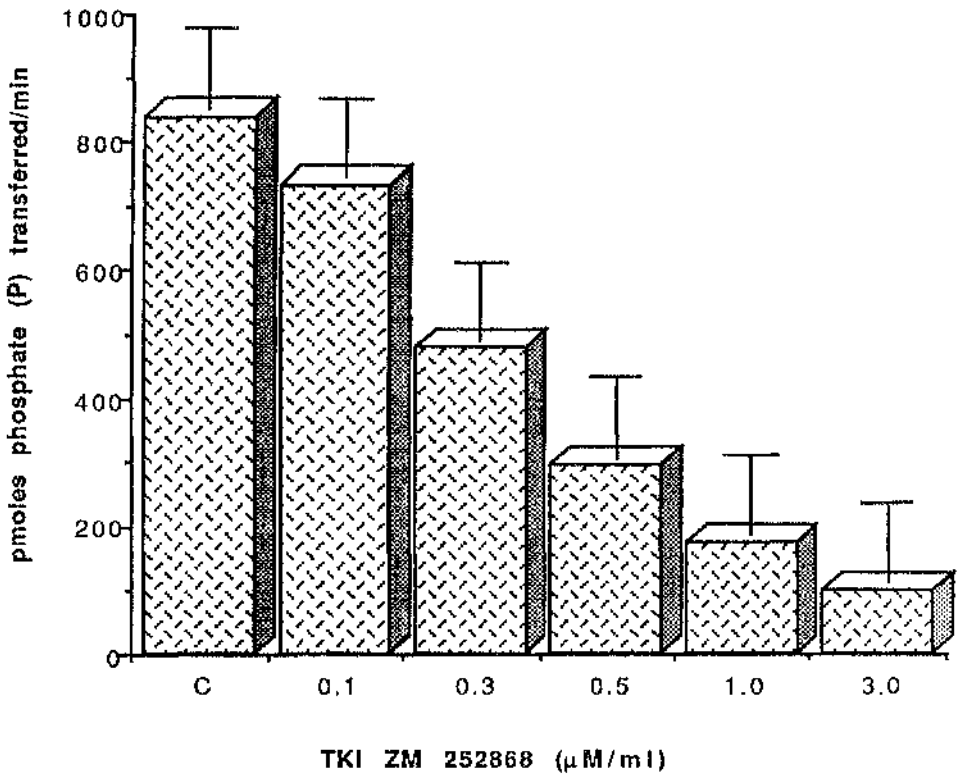
Inhibition of enzyme activity of EGF-receptor tyrosine kinase by ZM (252868) is expressed in the form of dose response curve. Fig. 29 illustrates the dose response of OAW42 cells. These are shown as means of 3 independent experiments and the standard error of the mean are indicated by the error bars.

Figure 29 shows the inhibition of OAW42 tyrosine kinase enzyme activity by increasing concentration of ZM from (0.1 to 3.0 μM) reached a maximum of a significant inhibition about 88% below control cell enzyme activity at the highest dose of ZM ($p = 0.0008$).

This indicates that blocking EGFr activity could be a possible therapeutic approach for EGFr +ve ovarian cancer.

Figure 29. The effect of tyrosine Kinase inhibitor ZM (252868) on EGF-receptor enzyme activity secreted by OAW42 cells. Cells were exposed for 30 minutes to increasing concentrations of ZM inhibitor (0.1 to 3.0 μM). Values represent SEM (bars) of triplicate experiments.

Inhibition of EGF receptor tyrosine kinase by ZM 252868 in OAW42 cells



3.12 GROWTH RESPONSE TO TKI

The effect of various concentration of Zeneca Inhibitor (ZM 252868) on growth of ovarian cancer cell lines OAW42, PEO1, and PEO1cddp.

Method

All three cell lines were subcultured as in section 2.4.3. of Methods chapter so that a 20% monolayer was formed within 3x96 well tissue culture plates per cell line as in section 2.7.2. of Methods Section. The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (Phenol Red-Free Medium) supplemented with 5% DHIDCCFCS in the presence of increasing concentrations of TKI (ZM 252868) 0.1 to 3.0 μM. 8 wells were used for each concentration of TKI. Control cells were incubated in experimental medium without additive. The cells were incubated at 37°C for 72 hours with changes of experimental medium +/- TKI at 48 hours. Every 24 hours incubation, one plate for each condition was treated by adding 50 μl MTT dye (concentration 5 mg/ml in sterilised PBS) and the MTT assay carried out as described in section 2.7.3. of the Methods chapter.

Results

The results of the TKI sensitivity studies are expressed in the form of time course and dose response for OAW42, PEO1, and PEO1cddp cells. These are shown as means of 3 independent experiments and the standard error of the means are indicated by the error bars.

There was no significant inhibition of OAW42 cells over 24 hours fig. 30. After 48 hours, significant inhibition was not observed until a dose of 1.0 μM was matched. reaching a maximum of 48.4% ($p < 0.0001$) below control cell growth at 3.0 μM dose. After 72 hours exposure, cell proliferation was inhibited to 27.5% ($p < 0.0001$), and 38.3% ($p < 0.0001$) below control cell growth at 1.0, and 3.0 μM dose respectively.

Figure 31 shows the effect of TKI on growth of PEO1 ovarian cancer cell line. After 24 hours exposure, data showed a slight rise in cell proliferation of 19.7% ($p < 0.02$) above control cell growth at 0.1 μM dose and inhibition to the cell proliferation at 3.0 μM dose of 22.6% ($p = 0.009$). After 48 hours exposure, cell proliferation was inhibited at all doses reaching a maximum of 40.3% ($p < 0.0001$) at 3.0 μM dose. After 72 hours exposure similar results were seen as in 48 hours exposure, reaching a maximum of 55.0% ($p < 0.0001$) below control cell growth.
Figure 32 shows the effect of TKI on growth of PEO1cddp ovarian cancer cell line. After 24 hours exposure cell proliferation was inhibited by increasing concentration of TKI reaching a maximum of 48.9% ($p < 0.0001$) below control cell growth at 3.0 μM. Similar results were seen after 48 hours exposure, reaching a maximum of 46.4% below control cell growth. Data after 72 hours exposure show highly decreased of cell proliferation at all TKI doses, reaching a maximum of 59.3% ($p < 0.0001$) below control cell growth at 3.0 μM.

Conclusion

The effect of TKI on OAW42 cells compared with PEO1 and PEO1cddp cells. As can be seen from the results, the effect of TKI on growth of OAW42 cells shows inhibition to the growth over 48, and 72 hours reaching a maximum of 48.4% at 3.0 μM dose.

PEO1 cells shows growth inhibition after 24, 48, and 72 hours reaching a maximum of 55% at 3.0 μM dose.

PEO1cddp cells shows growth inhibition after 24, 48, and 72 hours reaching a maximum of 59.3% at 3.0 μM dose.

This comparison illustrates that the three cell lines were growth inhibited by different doses of TKI, with the greatest effect seen in PEO1cddp cells. Hence, there is good support for the use of this TKI in treating drug resistant ovarian cancer.

Figure 30. Effect of TKI (ZM 252868) on the growth of OAW42 human ovarian cancer cell lines. Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of TKI (0.1 to 3.0 μM). Values represent mean +/- SEM (bars) of triplicate experiments. Control with out TKI.

Time course and dose response of OAW42 cells to TKI

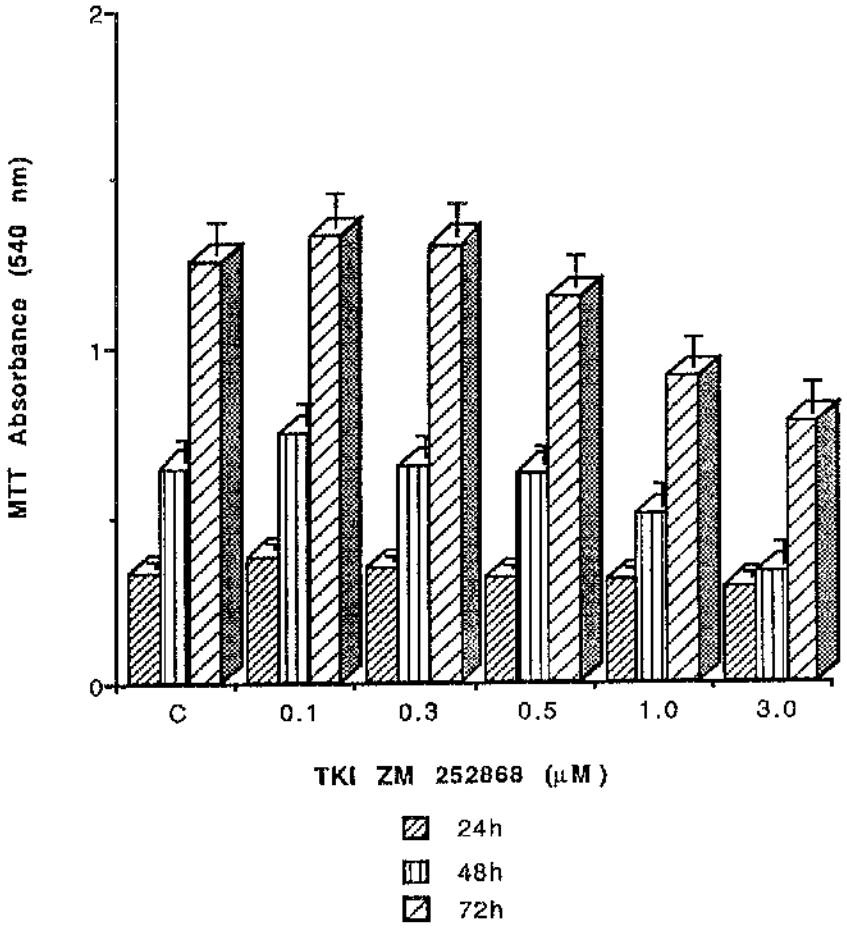


Figure 31. Effect of TKI (ZM 252868) on the growth of PEO1 human ovarian cancer cell line. Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of TKI (from 0.1 to 3.0 μM). Values represent mean +/- SEM (bars) of triplicate experiments. Control with out TKI.

Time course and dose response of PEO1 cells to TKI

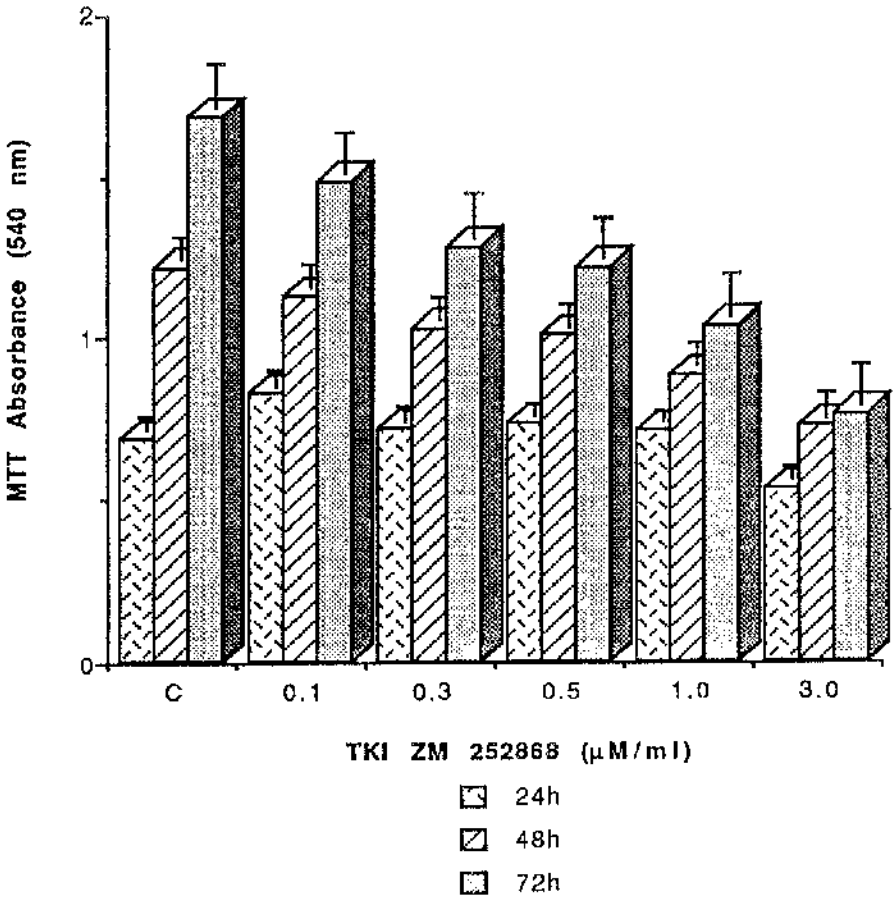
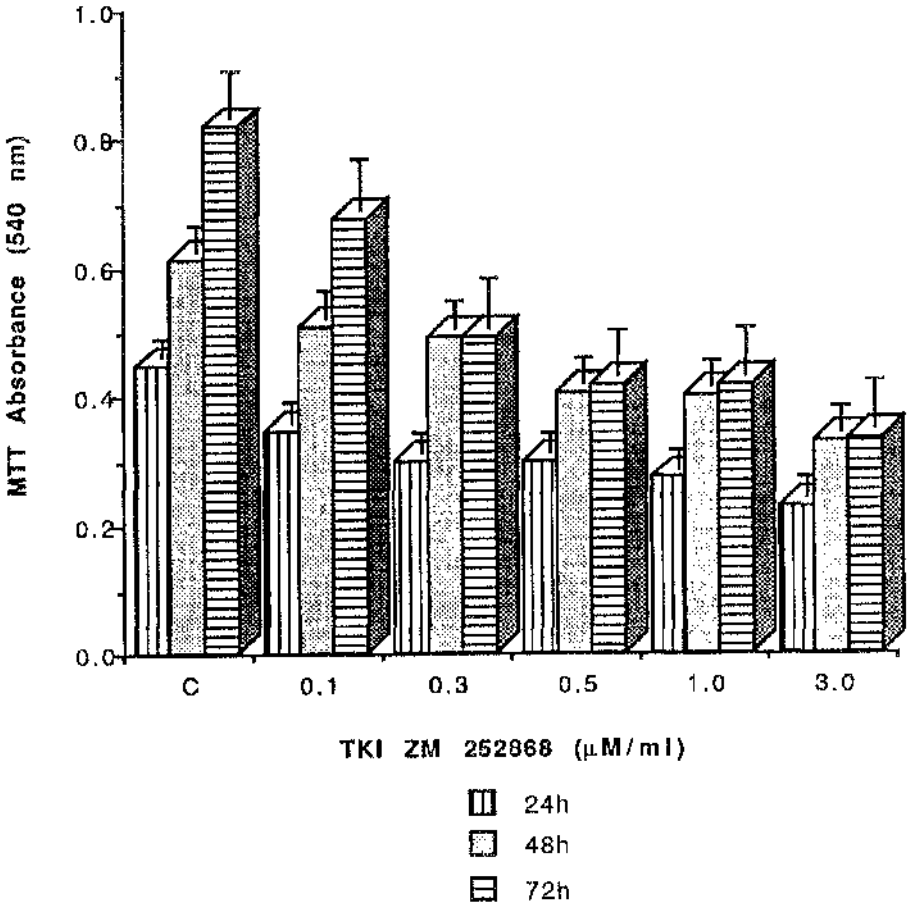


Figure 32. Effect of TKI (ZM 252868) on the growth of PEO1cddp human ovarian cancer cell line. Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of TKI (0.1 to 3.0 μM). Values represent mean +/- SEM (bars) of triplicate experiments. Control with out TKI.

Time course and dose response of PEO1cddp cells to TKI



3.13 GROWTH RESPONSE TO A COMBINATION OF EGF+TKI

From the previous study it was concluded that TKI (ZM 25868) inhibited growth of all three cell lines. As the inhibitor is said to be specific for the EGF receptor, it was of interest to determine whether addition of a known inhibitory dose of TKI (0.5 μM) could, overcome the stimulation by EGF.

Method

The three cell lines were subcultured as in section 2.4.3. of the Methods chapter. Cells were plated down using 3x96 well tissue culture plates per cell line as in section 2.7.2. of the Methods chapter. The cells were then allowed to plate down for 24 hours before the routine medium was removed and the cells washed twice with PBS. Experimental medium (Phenol Red-Free Medium) supplemented with 5% DHIDCCFCS in the presence of increasing concentrations of EGF plus a fixed level of TKI (ZM 252868, 0.5 μM). Control cells were incubated in experimental medium without additive. The cells were incubated at 37°C for up to 72 hours with a change of experimental medium after 48 hours incubation. After every 24 hours exposure, one plate of each cell line was treated by adding 50 μl MTT dye (concentration 5 mg/ml in sterilised PBS). The MTT assay was carried out as described in section 2.7.3 of the Methods chapter.

Results

The results of the effect of EGF + TKI on growth of ovarian cell lines are expressed in the form of time course and dose response. These are shown as the mean of 3 independent experiments and the standard error of the means are indicated by the error bars.

Figure 33 shows the effect of different doses of EGF + TKI (0.5 μM) on growth of OAW42 cells. After 24 hours incubation data shows stimulation to the growth reaching a maximum of 19.2% ($p < 0.0001$) at 2.5 ng/ml dose. After 48 hours incubation data shows slight increase in cell proliferation was observed reaching a maximum of 8.4% ($p = 0.003$) at 5 ng/ml dose, inhibition to the growth was seen in the other doses reaching a maximum of 11.2% ($p = 0.02$) below control cell growth at 50 ng/ml dose. After 72 hours exposure cell proliferation was inhibited by the effect of EGF + TKI reaching a maximum of 20.3% ($p = 0.0001$) at 50 ng/ml dose.

Figure 34 shows the effect of different doses of EGF + TKI (0.5 μM) on growth of PEO1 cells. After 24 hours exposure data shows inhibition to the growth reaching a maximum of 34% ($p = 0.005$) at 50 ng/ml dose. After 48 hours exposure data shows a small stimulation at doses up to 10 ng/ml and growth inhibition was seen at high dose reaching a maximum of 17.8% ($p = 0.2$) below control cell growth. After 72 hours incubation an increase in cell proliferation reaching a maximum of 28% ($p = 0.008$) above control cell growth at 2.5 ng/ml dose. Figure 35 shows the effect of different doses of EGF + TKI (0.5 μM) on growth of PEO1cddp cells. After 24 hours exposure data showed inhibition to the growth reaching a maximum of 31% ($p = 0.007$) below control at 50 ng/ml dose. After 48 and 72 hours exposure data showed inhibition to the growth reaching a maximum of 19 and 23% ($p = 0.4$) below control cell growth at 50 ng/ml dose respectively.

Conclusion

The effect of EGF + TKI on growth of three cell lines OAW42, PEO1, and PEO1cddp cells compared with effect of EGF alone. As can seen from figure 33 the effect of different doses of EGF + TKI (0.5 μM) on growth of OAW42 cells. After 24 hours incubation there was slight increase in cell stimulation was observed compared to the different doses of EGF alone (figure 11). After 48 and 72 hours a slight decrease in the cell growth was observed compared to the effect of different doses of EGF alone.

Figure 34 shows the effect of different doses of EGF + TKI (0.5 μM) on growth of PEO1 cells. After 24 hours incubation a decrease in cell proliferation was seen compared to the EGF alone. After 48 and 72 hours there was an increase in cell proliferation compared to the effect of the same doses of EGF alone (figure 12).

Figure 35 shows the effect of different doses of EGF + TKI (0.5 μM) on growth of PEO1cddp cells. There was no effect seen in cell proliferation after 24, 48, and 72 hours incubation compared to the effect of the same doses of EGF alone (figure 13) This comparison illustrates that more inhibition to the tyrosine kinase of EGFr in OAW42 and PEO1 cells than PEO1cddp cell line due to the effect of TKI dose.

Figure 33. Effect of EGF + TKI (ZM 252868) on the growth of OAW42 human ovarian cancer cell line. Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of EGF (From 2.5 to 50 ng/ml) + TKI (0.5 μM) Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of OAW42 cells to EGF + TKI

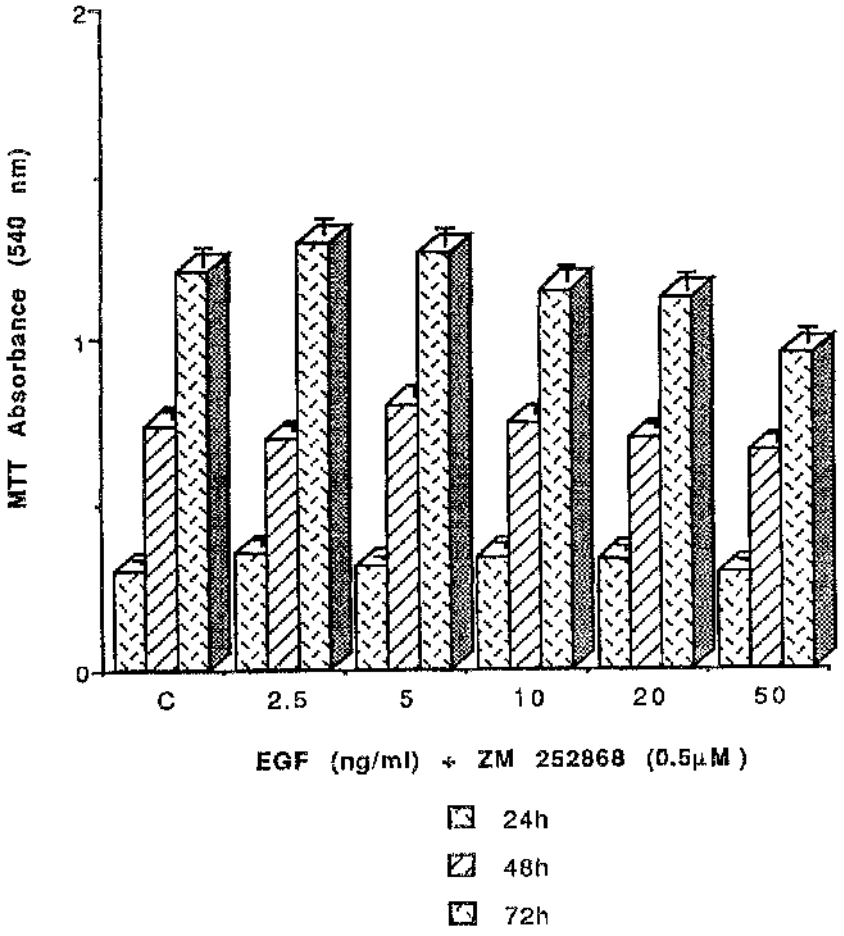


Figure 34. Effect of EGF + TKI (ZM 252868) on the growth of PEO1 human ovarian cancer cell line. Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of EGF (2.5 to 50 ng/ml) + TKI (0.5 μM). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1 cells to EGF + TKI

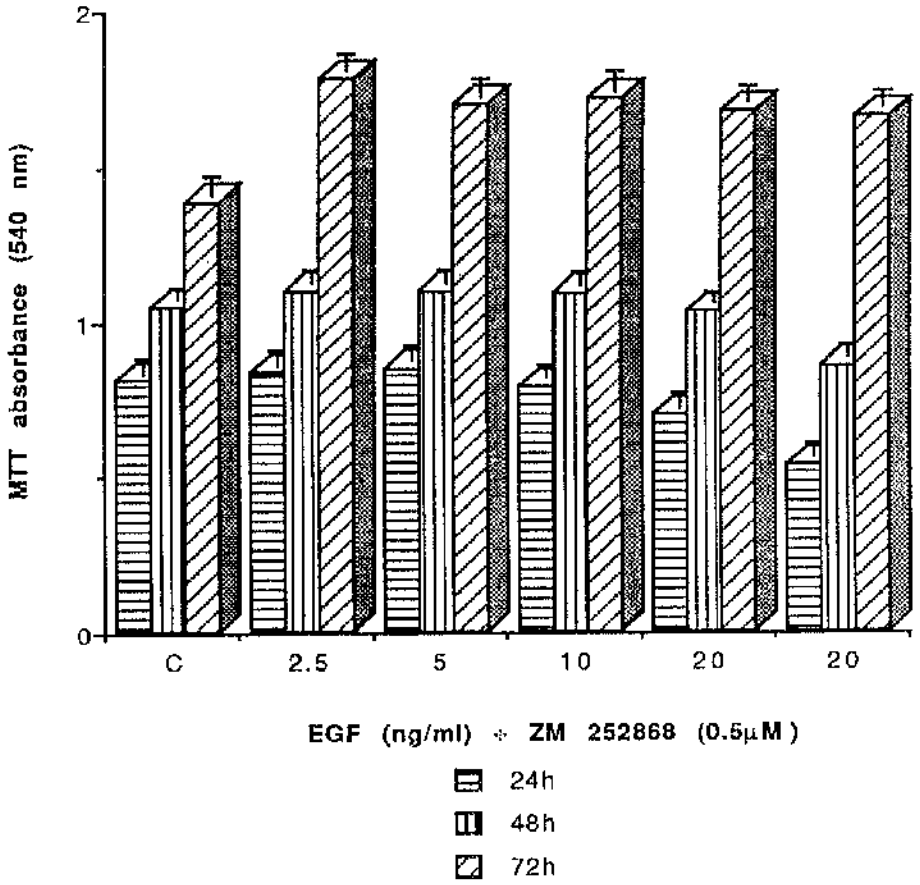
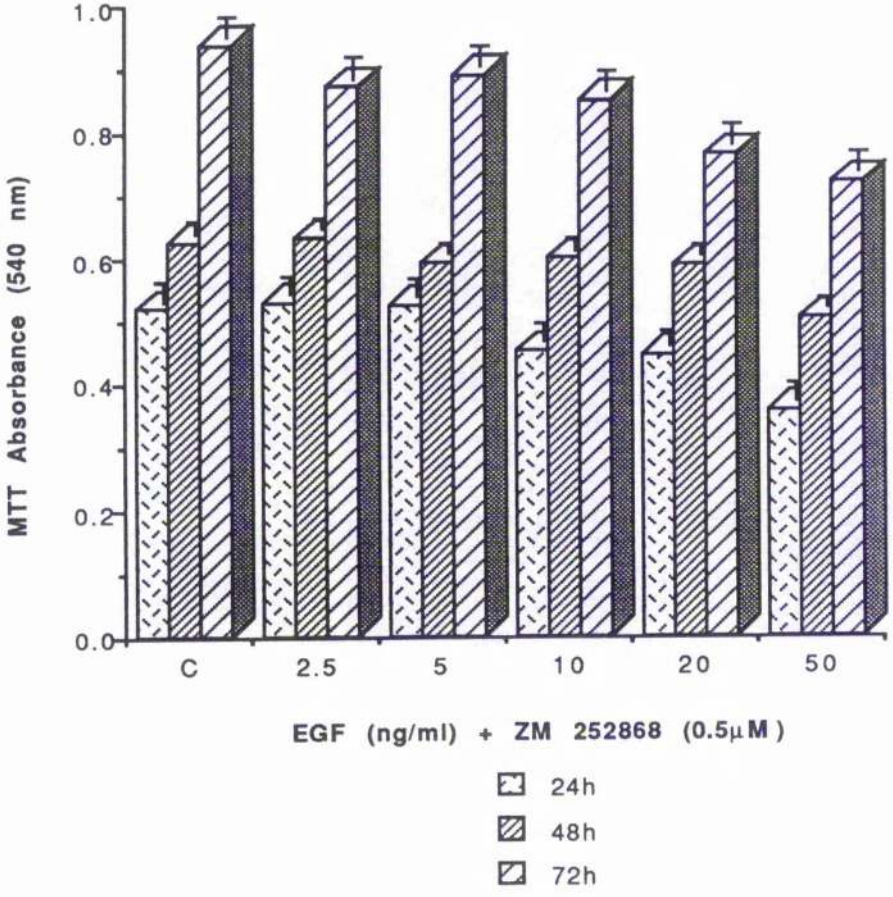


Figure 35. Effect of EGF + TKI (ZM 252868) on the growth of PEO1cddp human ovarian cancer cell line. Cells were grown in experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS and exposed for 24, 48 or 72 hours to increasing concentrations of EGF (2.5 to 50 ng/ml) + TKI (0.5 μM). Values represent mean +/- SEM (bars) of triplicate experiments.

Time course and dose response of PEO1cddp cells to EGF+TKI



3.14 MORPHOLOGICAL CHANGE OF OAW42

Study of the changes of morphology of OAW42 ovarian cancer cells, in response to the effects of LH and EGF, in comparison to control, was carried out by electron microscopy.

Method

Exponentially growing cells were harvested by trypsinization and plated down in 4x25 cm^2 tissue culture flasks at densities of approximately $5x10^4$ cells per flask, in RPMI 1640 containing 10% FCS. After 24 hours incubation, to allow the cells to attach, the medium was removed and cells washed twice with PBS. Experimental medium (Phenol Red Free Medium) supplemented with 5% DHIDCCFCS in the presence of LH concentration (40 iu/l) and/or EGF concentration (10 ng/ml) was then added. Control cells were incubated in experimental medium without additive. The cells were incubated at 37°C for 72 hours with a change of experimental medium at 48 hours. Experimental medium was removed, cells were washed with PBS and fixed in glutaraldehyde (see 2.10) and examined by electron microscopy.

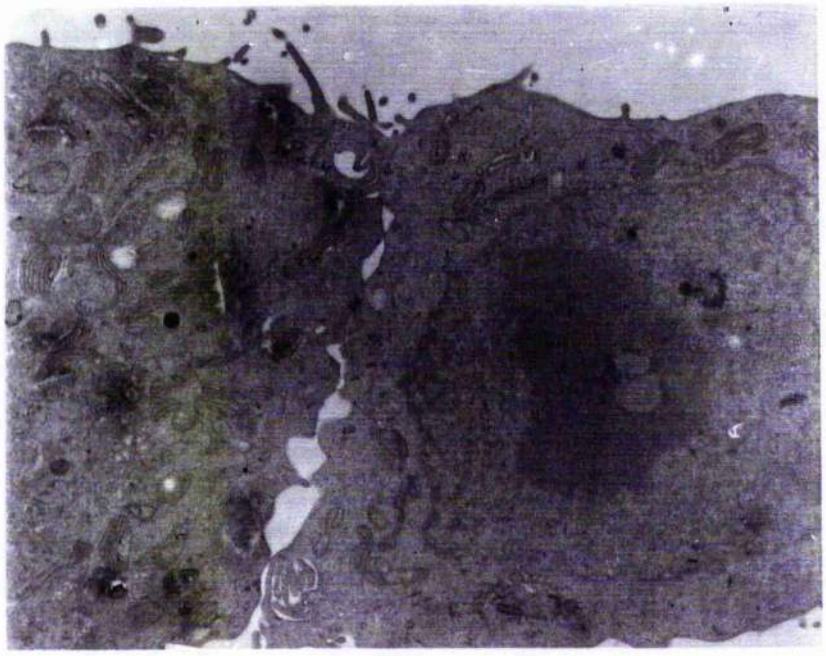
Results

The comparison has suggested a number of interesting similarities and differences (changes in size of mitochondria, changes in secretory activity) between cells treated for 72 hours with either LH or EGF. Figure 36 shows the transmission electron photomicrograph of OAW42 cells after exposure to EGF at 10 ng/ml up to 72 hours. Polysomes are more easily observed than in control cells; mitochondria are more swollen (and there is trend for a greater number in the EGF-treated cells); more stubby microvilli can be observed. In contrast, after 72 hours exposure to LH, there is no apparent swelling of the mitochondria, the cytoplasmic polysomes are less prominent than after exposure to EGF and the number of stubby microvilli was much reduced-being similar to that on control cells.

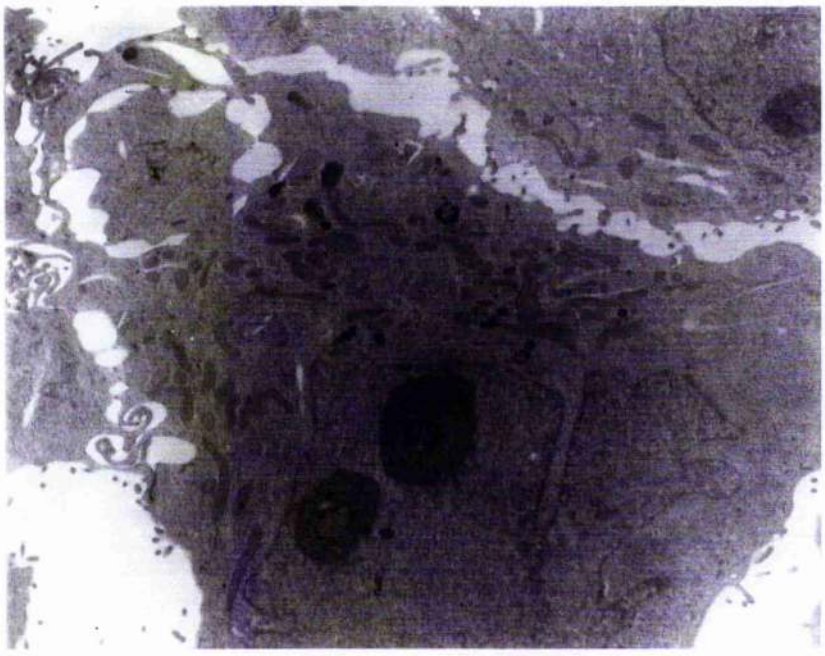
Conclusions

The effect of EGF on OAW42 cells was to cause mitochondrial swelling, very prominent polysomes and more stubby microvilli, all presumably reflecting the overall of growth response to the effect of EGF after 72 hours incubation. The effect of LH after 72 hours exposure shows no evidence for swelling of mitochondria, nor of cytoplasm-prominent polysomes and few stubby microvilli are seen. These results perhaps reflect the early growth inhibition by LH that was seen (figure 17) at 40 iu/l.

Figure 36. Electron microscopic examination for morphological change of OAW42 cell. Cells as control (A), Cells + LH (B), Cells + EGF (C) and Cells + LH and EGF (D). (11,457 magnifications).

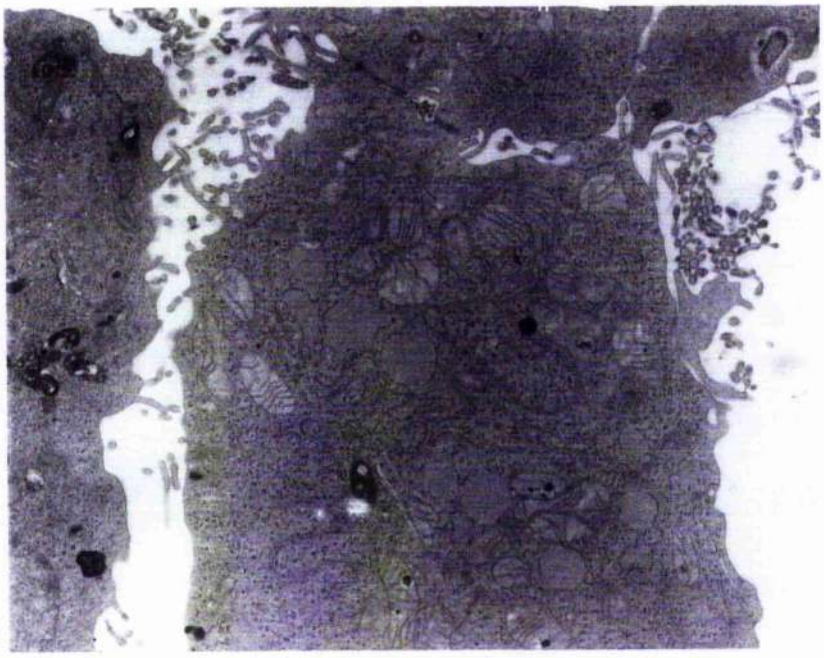


A



B

C



D

3.15 TGF-β1 WHOLE-CELL BINDING ASSAY

Radio receptor assay of TGF-β1 receptor in the OAW42 ovarian tumour cell line was carried out through incubation of cells with ^{125}I-TGF-β1 in the presence or absence of unlabelled 20 nM TGF-β1, following the procedure illustrated in section 2.10. of the Methods chapter.

Results

Counting efficiency = 40%

Specific activity = 1080 Ci/m mol

Total binding = 20706 cpm/200 µl aliquots of lysate cells

Non-specific binding = 15017 cpm/200 µl aliquots of lysate cells

Specific binding = Total binding - Non-specific binding

= 20706 - 15017

= 5689 cpm

1Bq = 1 disintegration/sec

1Ci = 3.7×10^{10} Bq

1 KBq = 27 nCi

5689 cpm = 5689/60 cps x 2.5 Mev

= 237 dps

= 237 Bq

= 0.237x27 = 6.4 nCi

1080 Ci/m mol

1.08×10^{3} Ci/m mol

6.4 nCi

6.4×10^{-9}Ci

6.4×10^{-9}Ci

1.08×10^{3}Ci/m m

$= 5.9x10^{-12}$ m mol

$= 5.9x10^{-15}$ mol

5.9 f mol/200 µl receptor binding buffer

29.5 f mol/ml

29.5 f mol receptor/ml

180 µg DNA/ml, (DNA was measured by using Hoechst DNA assay see (section 2.5.2) of the Methods chapter.

46 µg Protein/ml, (Protein was measured by using Bradford protein assay see (section 2.6.2) of the Methods chapter.

$$\frac{\text{Receptor fmol/ml}}{\text{DNA µg/ml}}$$

29.5/180 = 0.16 f mol Receptor/µg DNA

= 6,000 sites/cell

$$\frac{\text{Receptor f mol/ml}}{\text{Protein µg/ml}}$$

29.5/46 = 0.64 f mol Receptor/µg Protein

Conclusion

The presence of TGFβ binding activity in OAW42 cells has been demonstrated. From the previous result of dose response of cells to TGFβ, as seen from figure 16, growth inhibition of OAW42 cells occurred at all doses of TGFβ but the receptors appeared to be almost saturated at 5 ng/ml..

CHAPTER IV

DISCUSSION

DISCUSSION

Ovarian cancer arises almost exclusively in epithelial cells derived from the surface of the ovary. It is thought that the combination of repeated damage caused by ovulation (break of the epithelial surface layer to allow release of the follicle) and subsequent repair is the prime factor in the promotion of any transformed cells into ovarian epithelial cancer. Thus, stimulation by the factors involved in this wound repair could be critical. The most obvious candidate is epidermal growth factor (EGF) as the principal factor in all epithelial wound repairs.

EGF works through the EGF receptor (EGFr) and EGFr has been detected in about 45% of human ovarian epithelial cancer (Owens, *et al* 1991). Little is known about the regulation of EGFr in ovarian epithelial cells. Malignant cells may escape normal growth regulatory controls by secreting growth factors into their local environment (Todaro, *et al* 1980).
Production of growth factors by some tumours of epithelial origin has been characterized as autocrine stimulation (Browder, *et al* 1989). These growth factors may be necessary for optimal *in vivo* growth of the tumours (Wilson, *et al* 1991).

Normal human ovarian epithelial cells express both EGF and epidermal growth factor receptors (Scurry, *et al* 1994). However, EGF was detected in only 27.6% of samples of ovarian cancer whereas TGFα was present in 88.5% (Owens, *et al* 1991). Data have been presented in this thesis which demonstrate the importance of growth factors in determining proliferation of ovarian cancer cells and how growth regulation can be modulated on the one hand by TGFβ, and on the other hand by TGFα, EGF and a tyrosine kinase inhibitor which selectively blocks the EGF receptor.

The initial phase of this thesis described the control of cell proliferation using DNA and MTT assay methods based on the published method of Leake and Habib (1987) for DNA assay and Mossmann (1983) for MTT assay regarding to the effects of growth factors and hormones.

To establish whether ovarian cancer cell lines respond to exogenous peptide growth factors. OAW42 epithelial ovarian cancer cells (gift from Dr. A. Wilson), previously established from the ascites of a patient with ovarian cancer, were initially used. PEO1 and the platinum resistant PEO1cddp (gift from Dr. S. P. Langdon) were selected to further the study because they represent ovarian cancer cell lines from a patient whose tumour was initially sensitive to platinum but then became resistant to platinum therapy.

Growth response to EGF shows that OAW42 cells respond positively to the influence of EGF after 48 and 72 hours reaching a maximum of 34% and 21% above control at 10 ng/ml and 5 ng/ml respectively (as seen in figure 11). PEO1 cells showed earler stimulation to EGF, reaching a maximum of about 18.9% after 24 hours, 30.8% at 48 hours and 16.1% after 72 hours at the same dose of EGF (2.5 ng/ml) in contrast to the differences in maximum dose for TGFα.

The PEO1 cell line has also been reported by others to be growth stimulated at concentrations between 0.1 and 10 nmol/l (Crew *et al* 1992). This report found maximum stimulation at 1nmol/l, which compares with the 5 ng/ml seen in the experiments described here (1 nmol/l = 5.3ng/ml).
PEO1cddp cell growth responds to the direct influence of EGF. After 24 hours exposure there was a slight stimulation by 5 ng/ml (about 6.3% above control). After 48 hours there was no significant stimulation of growth seen and EGF inhibited growth at all doses after 72 hours. The EGFr may all be saturated with the ligand after 48 hours and probably this may be the reason why no stimulation was observed after 72 hours.

To investigate the balance of sensitivities to TGFα in both cell lines OAW42 and PEO1, the time course and dose response curves were determined (figure 14 and 15). These show the growth response to TGFα in OAW42 cells gave a slight rise of cell proliferation at a dose of 10 ng/ml at 24 hours incubation, whereas PEO1 showed a maximum of cell proliferation about 35% above control at 25 ng/ml dose. After 48 hours both cell lines were growth stimulated by effects of TGFα (10 and 50 ng/ml), reaching a maximum of 9.3 and 41.3% above control respectively, data after 72 hours showed a maximum of cell growth of 24.4% at 10 ng/ml.

Thus, although both cell lines respond to TGFα, the dose required to induce maximum response is significantly less in OAW42 than in PEO1 but the extent of stimulation of growth is much higher in PEO1. Compares with Crew values for maximum response to TGFα in between (0.1 and 10 nmol/l). In conclude OAW42 cells more sensitive to E2, PEO1 more sensitive to GF's.

TGF-β is a peptide growth factor that has been shown to inhibit proliferation of many normal and cancer epithelial cells. TGF-β inhibited proliferation of normal ovarian epithelial cells between 40-70% (Berchuck *et al* 1991). These data suggest that TGF-β might act as an autocrine growth inhibitor factor in normal ovarian epithelium. The OAW42 ovarian cancer cells were also markedly growth inhibited by 96 hours exposure to TGF-β1 (about 33.5 and 35.9%) at 1.0 and 5.0 ng/ml, respectively (as seen in figure 16).

These results contrast with the evidence from Hurteau *et al* (1994) who found that immortalized ovarian cancer cell lines were resistant to the growth inhibitory effect of TGFβ and frequently had lost the ability to produce or activate this growth factor.

In contrast, in their studies, TGF-β (10 ng/ml) significantly inhibited (3H) thymidine incorporation in 19 of 20 (95%) primary ovarian cancers. This would lend support to the idea that loss of inhibition by TGF-β is a critical step in the progression of ovarian cancer but it may also simply illustrate the differences that can be found among different ovarian cancer cell lines.

TGF-β is synthesised and secreted by the thecal cells. Nevertheless, thecal cells do not appear to have TGF-β receptors and the main target is thought to be the granulosa cells, though ovarian cancer cells in culture show marked growth inhibition by TGF-β, leading to the concept that a balance of control by TGF's α and β may regulate the progression of some ovarian cancers (Huteau, *et al* 1994, Bast *et al* 1995). Increased activity of TGFα and decreased activity of TGFβ may contribute to the development of many ovarian cancer. Loss of TGFβ responsiveness has been associated with the downregulation of c-myc expression in the development of ovarian cancer (Chow *et al* 1996).

Conclusions from these studies are that OAW42 and PEO1 ovarian cancer cells express EGF receptors and are responsive to EGF and TGFα. This supports the suggestion that the growth of certain ovarian cancers could be maintained by EGF through the EGF receptor (Owens *et al* 1991a). EGF receptor have been reported to be present in about 50% of ovarian tumours extracts (Bauknecht *et al* 1989) and (Leake *et al* 1990). TGFα has been found in 88% of ovarian cancers (Owens *et al* 1991b).
Therefore EGF and TGFα may have a role in autocrine and/or paracrine growth modulation of ovarian cancer.

The results showed that TGFβ inhibited cell proliferation and DNA synthesis in OAW42 cells. These results together with those from previous studies show that these cells express TGFβ, TGFα and EGF receptors and that overall cell growth may be modulated by a balance of these growth factors in an autocrine and/or paracrine manner.

Another example of the link between hormones and growth factors was provided by the study of Luteinizing hormone (LH) regulation of ovarian cancer cell growth. Growth response to LH, was studied at various concentrations. LH was shown to inhibit growth of both OAW42 and PEO1 cells. As seen in figures (17) and (18), by increasing the dose of LH there was an increase in cell inhibition, reaching a maximum, in the two cell lines, of about 21.3 and 55.5% respectively below control at 60 iu/l dose after 24 hours exposure of each cell line to LH. After 48 and 72 hours OAW42 cell growth exhibited a maximum inhibition reaching about 32% below control at 60 iu/l at both 48 and 72 hours. PEO1 showed greater inhibition (about 58.2 and 78.7% below control after 48 and 72 hours respectively)

The previous study showed that LH is capable of causing dose dependent inhibition of ovarian cancer cells at least *in vitro*. In order to determine whether LH increased the synthesis and secretion of active TGFβ into the cell culture medium, cells were co-incubated with both LH and commercially available anti-TGFβ polyclonal antibody at 5 and 30μl/ml. As is seen in figure (18), this antibody reversed the growth inhibition by LH and even allowed growth (11.3%) above control indicating that TGFβ partially suppresses OAW42 cell growth under control conditions. These results suggest that the anti-proliferation effects of LH on ovarian cancer cells are mediated by release of TGFβ. Therefore it is appears that TGFβ is a hormonally regulated growth inhibitor with possible autocrine function in ovarian cancer cells.

Presence of EGF receptor, has been confirmed by demonstrating the binding of iodinated EGF to total particulate fractions of the cell lines. Scatchard analysis of the ligand binding data indicated that OAW42 and PEO1 cells exhibited high affinity EGF receptor with a maximum binding capacity, 24.8 and 21.5 fmol/mg protein, and dissociation constant (Kd), 0.833 and 0.840 nM respectively as can seen in Table (3).

Cancer and other hyperproliferative diseases are associated with elevated protein tyrosine kinase activity, related to the increased activity of growth factor receptors. It has been hypothesized that chemical blockers, particularly of the substrate domain of the receptor protein tyrosine kinase, might prove to be effective and potentially selective inhibitors of cellular proliferation in diseases which are regulated by a cell growth factors and hormones (Arteaga, *et al* 1988, Osborne, *et al* 1976, and Arteaga, *et al* 1989).

The first class of tyrosine kinase inhibitors described, called tyrphostins, had some selectivity for the EGF receptor (Yaish, *et al* 1988). Further more, these tyrphostins were more potent inhibitors of EGF-stimulated cell proliferation than of serum- stimulated cell proliferation.

To confirm that selective inhibitors can block EGF receptor autophosphorylation in ovarian cancer cells, the effects of Zeneca inhibitor ZM 252868 on the OAW42 ovarian cancer EGF receptor positive cell lines were determined. After exposure of these cells to varying concentration of ZM 252868, EGF receptor tyrosine kinase enzyme activity demonstrated significant inhibition. Tyrosine phosphorylation of the EGF receptor was inhibited in a dose dependent manner (fig. 29), reaching a maximum of about 88% below control cell enzyme activity at the maximum dose (3 μM) of ZM inhibitor. This inhibition of TK activity was reflected in reduced growth, as shown in (figure 29) as the 88% inhibition of TK activity led to 60% growth inhibition after 72 hours.

It is possible that, in intact cells, the tyrosine kinase activity of other growth factor receptors or other tyrosine kinases critical to rapidly proliferation cells are also inhibited by this agent. Further studies are needed to show whether all the effects observed are mediated through the EGFr.

Other studies show that several tyrosine kinase inhibitors inhibit *in vivo* growth (Gillespie, *et al* 1993). Data from Fry, *et al* (1994) shows that the PD 153035 inhibited the EGF receptor tyrosine kinase by 50% below control (not as effective as the ZM 252868). Our data do show that tyrosine kinase inhibitor can inhibit proliferation of ovarian cancer cells.

Taking this into account, the present data raise the possibility that ZM 252868 may prove to be useful new agent for the treatment of ovarian tumours. Other work from our laboratory (Leake *et al* 1995) has shown that the EGF receptor TK activity is markedly elevated in PEO1cddp cells. If it can be confirmed that elevated EGFr TK activity is common in platinum resistant tumours, the use of inhibitor like ZM 252868 might be very promising for patients with drug resistant tumour. Other tumours may also be responsive to this a approach.

Electron microscopy was used, to study the hormonally-induced changes in the cellular morphology which occur as ovarian cancer cells form three-dimensional structures. EGF was studied because it is thought to be the major factor involved in the wound repair after ovulation.

The comparison has suggested a number of interesting similarities and differences in terms of changes associated with differentiation (changes in size of mitochondria, changes in secretory activity). Exposure of OAW42 cells to EGF at 10 ng/ml dose up to 72 hours cause mitochondrial swelling, very prominent polysomes and more stubby microvilli. Exposure of cells to LH at 40 iu/l after 72 hours incubation shows no evidence for swelling of mitochondria, nor of cytoplasm-prominent polysomes and few stubby microvilli are seen. These results reflect the growth promotion and inhibition which is induced in OAW42 cells by these two factors.

Conclusions

The study concerning the potential role of growth factors and hormones in the growth regulation of human ovarian cancer cells. Most ovarian cancers arise from single colonies of cells that have undergone multiple genetic alterations. Although most primary ovarian cancer cells remain sensitive to the growth-inhibitory effect of transforming growth factor-beta (TGF-β), loss of production may interrupt the transforming growth factor-beta autocrine

inhibitory loop and play a role in the development of some ovarian cancer. The inhibitory effects exerted by both LH and TGF-β on the proliferation of OAW42 cells clearly indicate these cells retain both their autocrine and paracrine growth inhibition by TGF-β.

Stimulatory effects by both growth factors TGFα and EGF on the proliferation of OAW42 and PEO1 ovarian cancer cells were demonstrated. Both cell lines OAW42 and PEO1 were shown to be EGF receptor positive. Therefore EGF and TGFα can be autocrine and/or paracrine growth factors in ovarian cancer.

Ovarian cancer arises from promotion of transformed epithelial cells by TGFα/EGF, acting at the sites of ovulatory damage. This promotion is mediated through the EGF receptor. Tyrosine kinase inhibitor inhibit proliferation of epithelial ovarian cancer cells at dose over 1.0 μM. Inhibition of the EGFr-associated T.K. can block this growth stimulation. As platinum resistant cells have elevated EGFr-TK, this may be a route for therapy for patient-resistant tumours. LH inhibits ovarian cancer cell growth through modulation of TGF-β function. As ovarian cancer progresses a loss of inhibition by TGF-β may be intical step in tumour progression. These hormonal and growth factor effects are reflected in changes induced in cellular morphology of mitochondria, microvilli and polysomes.

References

Adam, H. O., Persson, I., Hoover, R., Schairer, C., Bergkvist, L. 1989.
Risk of cancer in women receiving hormone replacement therapy.
Int J Cancer ; **44**: 833-839

Adashi, E.Y., Resnisk, C. E., Hernandez, E. R., May, J. V., purchio, A. F., and Twardzik, D. R. 1989.
Ovarian transforming growth factor-β (TGF-β): cellular site (s), and mechanism (s) of action.
Mol Cell Endocrine ; **61**: 247-256

Alders, J.G., and Neijt, J. P. 1993.
Aspects surgery in ovarian and endometrial carcinoma.
Eur J Cancer Vol **29** A ; 4: 624-628

Alice, S., Whittemore, Robin Harris, Jacqueline Ltnyre, and the collaborative ovarian cancer group. 1992.
Characteristics relating to ovarian cancer risk collaborative analysis of 12 US case-control studies.
AM J Epidemiol ;**136**: 1184-1203

Anzano, M. A., Roberts, A. B., Delarco, J. E., Wakefield, L. M., Assoian, R. K., Roche, N. S., Smith, J. E., Lazarus, J. E., and Sporn, M. B. 1985.
Increased secretion of type β transforming growth factor accompanies viral transformation of cells.
Mol Cell Biol ; **5**: 242-247

Arteaga, C. L., and Osborne, C. K. 1989.
Growth inhibition of breast cancer cells *in vitro* with an antibody against the Type 1 somatomedin receptor.
Cancer Res ; **49** : 6237-6241

Arteaga, C. L., Coronado, E., and Osborne, C. K. 1988.
Blockade of the epidermal growth factor receptor inhibits transforming growth factor alpha-induced but not oestrogen-induced growth of hormone-dependent human breast cancer.
Mol Endocrinol ; **2** : 1064-1069

Arteaga, C. L., Hananke, A. R., Clark, G.M., Osborne, K., Hazarika, P., Pardue, R. L., Tio, F., Von Hoff, D. D. 1988.
Immunoreactive-α transforming growth factor activity in effusion from cancer patients as a marker of tumour burden and patient prognosis.
Cancer Res ; **48**: 5023- 5028

Asem, E. K., Carnegie, J. A., Tsang, B. K. 1992.
Fibronectin production by chicken granulosa cells in vitro: effect of follicular development.
Acta Endocrinology (Copenh) **127**: 466-470

Bast, R. C., Klug, T. L., St. John, E., Jenison, E., Niloff, J. M., Lazarus, H., et al 1983. A radioimmunoassay using a monoclonal anti-body to monitor the course of epithelial ovarian cancer.
N Engl J Med ; **309** : 883-887

Bast,R. C., Boyer, C. M., Feng Xu, Wiener, J., Dabel, R., Woolas, R., Jaccobs, I., Berchuck, A. 1995.
Molecular approaches to prevention and detection of epithelial ovarian cancer.
Journal of Cellular Biochemistry ; **59** : 219-222

Bauknecht, T., Birmelin, G., Kommoss, F. 1990.
Clinical significance of oncogenes and growth factors in ovarian carcinomas.
J Steroid Biochem Mol Biol ; **37** : 855-862

Bauknecht, T, Kohler, M., Janz, I., and Pfleiderer, A. 1989.
The occurrence of epidermal growth factor receptor and the charactterization of EGF-Like factors in human ovarian endometrial, cervical and breast cancer.
J Cancer Res Clin Oncol ; **115** : 193-199

Bauknecht, T., Runge, M., Schwall, M., Pfeiderer, A., 1988.
Occurrence of epidermal growth factor receptors in human adnexal tumours and their prognostic value in advanced ovarian carcinomas.
Gynecol Oncol ; **29**:147-157

Beers, W. H., Strickland, S. and Reich, E. 1985.
Ovarian plasminogen activator, relationship to ovulation and hormonal regulation.
Cell ; **6** : 387-394

Bendell, J. J., and Dorrington, J. 1988.
Rat thecal /interstitial cells secrete a transforming growth factor-β like factor that promotes growth and differention in rat granulosa cells.
Endocrinology ; **123**: 941-948

Berchuck, A. 1995.
Biomarkers in the ovary.
Journal of Cellular Biochemistry ; **59** : 223-226

Berchuck, A., Kamel, A., Whitaker, R., et al. 1990.
Overexpression of *HER -2/neu* is associated with poor survival in advanced epithelial ovarian cancer.
Cancer Res ; **50** : 4087-4091

Berchuck, A., Olt, G. J., Everitt, L., Soisson, A. P., Bast, R. C., and Boyer, C. M. 1990.
The role of peptide growth factor in epithelial ovarian cancer.
Obstes Gynecol ; **75**: 255-261

Berchuck, A., Rodriguez, G. C., Olt, G. L., Boente, M. P., Whitaker, R. S., Arrick, B.A., Clarke -Pearson, D. L., Bast, R. C.1991.
Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by TGF-β.
AM J Obstet Gynecol ; **166**: 676-684

Berek J. S. 1989.
Epithelial ovarian cancer In: Berek, J. S., Hacker, N. F, eds. Practical gynecologic oncology. Baltimore: Williams and Wilkins; 327-364

Bishop, J. M. 1987.
The Molecular genetics of cancer.
Science ; **235** : 305-311

Bjersing, L., Cajanders. 1975.
Ovulation and the role of the ovarian surface epithelium.
Experiential ; **31**: 605-608

Blair, E. L., Kim, I. C., Estes, J. E., Keski-ojaj, Schomberg, D. W.1988.
Human PDGF preparations contain a separate activity which potentates FSH-mediated induction of LH/HCG receptor in cultured rat granulosa cells: evidence for transforming growth factor-β.
Endocrinology ;**123** : 2003-2008

Boring, C. C., Squires, T. S., Tong, T. 1992
Cancer statistics.
CA - A Cancer Journal For Clinicians ; **42**: 19-38

Bradford, M. M., 1976.
A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.
Anal Biochem ; **53**: 304-308

Browder, T. M., Dunbar, C.E., and Niehuis, A.W. 1989.
Private and public autocrine loops in neoplastic cells.
Cancer Cells ; **1**: 9-17

Brown-Shimer, S., Johnson, K. A., Hill, D. E., and Bruskin, A. M. 1992.
Effect of protein tyrosine phosphates 1B expression on transformation by the human *neu* oncogene.
Cancer Res ; **52** : 478-482

Buell, P., Dunn, J. E. 1965, 1968.
Cancer mortality among Japanese isseiand nisei of California.
Cancer ; **18**: 656-664

Burgess, A. W. 1989,
Epidermal growth factor and transforming growth factor-α.
By Med Bull ; **45**: 40-42

Cahill, L. P., Driancout, M. A., Chamley, W. A., and Findlay, J. K. 1985.
Role of intracellular regulators and FSH in growth and development of large antral follicles in sheep.
Journal of Reproduction and Fertility ; **75** : 599- 607

Carson, R. S., Robertson, D. M., and Findlay, J. K. 1988.
Ovarian follicular fluid inhibits thymidine incorporation by 3T3 fibroblasts in vitro.
Jurnal of Reproduction and Fertility ; **82** : 447-455

Chow, S. N., Chin, C. H., Chin, C. T. 1996.
Molecular biology of human ovarian cancer.
International Surgery ; **81** : 152-157

Cohen, S., Carpenter, G., and King, L. 1980.
Epidermal growth factor-receptor-protein Cannes interactions.
J Boil Chem ; **255**: 4834-4842

Cramer, D. W., Welch, W. R. 1983.
Determinants of ovarian cancer risk inferences regarding pathogenesis.
J Natal Cancer Ins ; **71**: 717-721

Crew, A. J., Langdon, S. P., Miller, E. P., and Miller, W. R. 1992.
Mitogenic effects of epidermal growth factor and transforming growth factor-alpha on EGF-receptor positive human ovarian carcinoma cell lines.
European Journal of Cancer ; **28**: 337-341

Davis, H. M., Zurawski, V. R., Bast, R. C. Klug, T. L.1986.
Characterization of the CA 125 antigen associated with epithelial ovarian carcinomas.
Cancer Res ; **46**: 6143-6148

Delarco, J. E., Todaro, G. J. 1986.
Growth factors from murine sarcoma virus-transformed cells.
Proc Natal Acid Sci USA ; **75** : No 8, 4001-4005

Derynck, R., Jarrett, J. A., Chen, E. Y., and Goedded, D.V.1986.
The marine transforming growth factor-β precursor.
J Biol Chem ; **261**: 4377- 4379

Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B, Sporn, M. B., and Goedded, D. V. 1985.
Human transforming growth factor-B cDNA sequence and expression in tumour cell lines.
Nature ; **316** : 701-705

Dierschke, D. J., Yamaji, T., Karsch, F. J., Wick, R. F., Wess, G., and Knobil, E. 1973.
Blockade by progesterone of estrogen-induced LH and FSH release in the rhesus monkey.
Endocrinology ; **92**: 1496-1501

Dierschke, D. J., Weiss, G. and knobil, E. 1974.
Sexual maturation in the female rhesus monkey and the development of oestrogen - induced gonadotrophic hormone release.
Endocrinology ; **94**: 198-206

Dodson, W. C., Schomberg, D. W. 1987.
The effect of transforming growth factor-β on follicle-stmiulating hormone-induced differentiation of cultured rat granulosa cells.
Endocrinology ; **120**: 512-516

Downward, J., Parker, P., and Waterfield, M. D. 1984.
Autophosphorylation sites on the epidermal growth factor receptor.
Nature ; **311**: 483-485

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlesinger, J., and Water field, M. D. 1984.
close similarity of epidermal growth factor receptor and v-*erb* B oncogenes protein sequences.
Nature ; **307**: 521-527

Easton, D. F., Bishop, D. T., Ford, *et al* 1993.
Genetic linkage analysis in familial breast and ovarian cancer-results from 214 families.
Am J Hum Genet ; **52** : 678-701

Eccles, D. M., Cranston, G. Steel, C. M., Nakamura, Y., and Leonard, R. C. F. 1990.
Allele losses on chromosome 17 in human epithelial ovarian carcinoma.
Oncogene ; **5** : 1599-1601

Faizuddin, M., Sen, N. K., El-Kassaby, S. M., Wahaba, M. H. 1989.
Prevalence of ovarian tumours.
Al Fateh Medical Journal ; **4**: No 1, 47-49

Fathalla, M. F. 1971.
Incessant ovulation- a factor in ovarian neoplasia.
Lancet ; **2** : 163-164

Feunteun,J., Narod, S. A., Lynch, H. T. *et al* 1993.
Abreast-ovarian susceptibility gene maps to chromosome 17q21.
Am J Hum Genet. ; **52**, 736-742

Findlay, J. K., and Risbridger, G. P. 1987.
Intragonadal control mechanisms.
Bailliere's Clinical Endocrinology a nd Metabolism ; **1** (1): 223-243

Fraumeni, J. J., Grundy, G. w., Creagan, E. T., Everson, R. B. 1974.
Six families porne to ovarian cancer.
Cancer ; **36**: 364-369

Fry, D. W., Kraker, A. J., Amy McMichael, Ambroso, L. A., Nelson, J. M., Leopold, W. R., Connors, R. W., Bridges, A. J., 1994.
A specific inhibitor of the epidermal growth factor receptor tyrosine kinase.
Science ; **265**: 1093-1095

Gillespie, J., Dye, J. F., Schachter, M., Guillou, P. J., 1993.
Inhibit of pancreatic cancer cell growth *in vitro* by the tyrphostin group of tyrosine kinase inhibitors.
Br J Cancer ; **68**: 1122-1126

Greene, M. H., Clark, J. W., Blayney, D. W. 1984.
The epidemiology of ovarian cancer.
Semin Oncol ; 11: 209-226

Gwinn, M. L., Lee, N. C., Rhodes, P. H. 1990.
Pregnancy, breast feeding, and oral contraceptives and the risk of epithelial ovarian cancer.
J Clin Epidemiol ; **43** : 559-568

Harding, M., Cowan, S., Hole, D., Cassidy, L., Kitchener, H., Davis, J., Leak, R., 1990.
Oestrogen and progesterone receptors in ovarian cancer.
Cancer ; **65** : 486-491

Hartge, P., Schiffman, M. H., Hoover, R. 1989.
A case-control study of epithelial ovarian cancer.
A M J Obstet Gynecol ;**161**:10-16

Hamilton, T. C., Davies, P., Griffith, K. 1992.
Oestrogen receptor-like binding in the surface germinal epithelium of the rat ovary.
J Endocrinol ; **95** : 377-385

Hankinson, S. E., Colditz, G. A., Hunter, D. J. Spencer, T. L., Rosner, B., Stampfer, M. J. 1992.
A quantitative assessment of oral contraceptive use and risk of epithelial ovarian cancer.
Obstet Gynecol ; **80**: 708-714

Havrilesky, L. J., Hurteau, J. A., Whitaker, R. S., Elbendary, A, Wu, S., Rodriguez, G. C., Bast, Rc,Jr., and Berchuck, A. 1995.
Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor beta.
Cancer Research ; **55** : 944-948

Hiramatsu, S., Maruo, T., Matsuo, H., Mochizuki, M. 1992.
Effects of epidermal growth factor on the proliferation and differentiation of porcine granulosa cells cultured in vitro.
Acta Obstet Gynecol ; **44**: 55-61

Hole, D., Gills, C. R., Still, R. M., Davies, J., Kaye, S. B.1991.
Medical audit cancer registration and survival in ovarian cancer.
Lancet ; **337**: 611-612

Hoover, R., Gray, L. A., Fraumini, J. F., 1977.
Stilbestrol and the risk of ovarian cancer.
Lancet ; **2**: 533-534

Hurteau, J., Rodriguez, G. C., Whitaker, R. S., Shah, S., Mills, G., Bast,R. C., Berchuck, A. 1994.
Transforming growth factor-beta inhibits proliferation of human ovarian cancer cells obtained from ascites.
Cancer ; **74**: 93-99

Jacobs, I., Bast, R. C. 1989.
The CA 125 tumour-associated antigen a review of the literature.
Hum Reprod ; 4 : 1-12

Jacobs, I. J., Oram, D. A. Potential screening test for ovarian cancer. In: Sharp, F., Mason, W. P., Leake, R. E, eds: 1990.
Ovarian cancer: biological and therapeutic challenges. London: Chapman and Hall Medical ; **20**: 197-205

Jely, D. J., Lilien-feld, A. M., Diamond, E. L., Bross, I. D. 1974.
An epidermiolgic study of the relationship of reproductive experience to cancer of the ovary.
A M J Epidemiol ; **99** : 190-209

Jhappan, C., Stahle, C., Harkins, R. N., Fausto, N., Smith, G. H., and Merlino, G. T. 1990.
TGF-α over expression in transgenic mice: induces liver neoplasia and abnormal development of the mammary gland and pancreas.
Cell ; **61**: 1137-1146

Ken-Ichiro Morishige, Hirohisa Kurachi, Kyoka Amemiya, Yoshiko Fujita, Toshiya Yamamoto, Akira Miyake, and Osamu Tanizawa. 1991.
Evidence for the involvement of transforming growth mechanism in primary human ovarian cancers.
Cancer Res ; **51** : 5322-5328

Kerlikowske, K., Brown, J. S., and Grady, D. G. 1992.
Should women with familial ovarian cancer undergo prophylactic oophorectomy.
Obstet Gynecol. ; **80** : 700-710

Koch, M., Gaedke, H., Jenkins, H. 1989.
Family history of ovarian cancer patients: A case-control study.
Int J Epidemiol ; **18**: 782-785

Kohler, M., Janz, I., Wintzer, H. O, Wagner, E., and Bauknecht, T. 1989.
The expression of EGF-receptors, EGF-Like factors and c-*myc* in ovarian and cervical carcinomas and their potential clinical significance.
Anti Cancer Res ; **9**: 1537-1547

Kurt, Stromberg, Theodore, J., Collins, I. V., Alfred, W., Gordon, Carolynl, L., Jackson. and Gibbesr, R., Johnson. 1992.
Acts of TGF-α in ovarian carcinoma cell lines.
Cancer Res ; **52**: 341-347

Kvale, G. Heuch, I., Nielson, S., Beral, V. 1988.
Reproductive factors and risk of ovarian cancer, A prospective study.
Int J Cancer ; **42**: 246-251

Langdon, S. P., Lawrie. S. S., Hay. F. G., Hawkes, M. M., McDonald, A., Hayward, I. P., Schol, D. J., Hilgers, J., Leonard, R. C. F., and Smyth, J. F. 1988.
Characterization and Properties of Nine Human Ovarian Adenocarcinoma cell lines.
Cancer Res ; **48**: 6166-6172

Leake, R., Barber, A., Owens, O., Langdon, S. and Miller, B. 1995.
Growth factors and receptors in ovarian cancer.
Ovarian cancer no 3, Frank Sharp, Peter Mason, Tony Blackett and Jonathan Berek. 99-108

Leake, R. E., and Habib, F. 1987.
Steroid hormone receptors: assay and characterization. In : Steroid Hormones: a Practical Approach. B .Green and R. E. Leake (ed.),
PP. 67 - 92. Oxford: IRL Press.

Leake, R. E., and Owens, O. 1990.
The prognostic value of steroid receptors, growth factors and growth factor receptors in ovarian cancer. In Sharp, F., Mason, W. P., Leake, R. E. eds. Ovarian Cancer. Biological and Therapeutic Challenges. London, Chapman and hall Medical. 69-75

Lee, D. C., Rochford, R. M., Tudor, G. T., Villareal, L. P. 1985.
Developmental expression of rat transforming growth factor-α mRNA.
Mol Cell Biol ; **5**: 3644-3646

Lewis, A. C. W., Davison, B. C. C . 1969.
Familial ovarian cancer.
Lancet ; **2**: 235-237

Lia, X., and Pawson, T. 1994.
Biochemistry of the Src protein-tyrosine kinase: regulation by Sh2 and SH3 domains.
Recent Prog Horm Res ; **49** : 149-160

Lynch, H. T., Kimberling, W., Albano, W. A. Lynch, J. F., Biscone, K., Schuelke, G. S., Sandberg, A. A., Lipkin, M., Deschner, E. E., Mikol, Y. B., Elston, R. C., Bailey -Wilson, J. E., Shannon, B.1985.
Hereditary non polyposis colorectal cancer (lynch syndrome I and II)
Cancer ; **56**: 934-938

Lynch, H. T., Bewtrac, Lynch, J. F. 1986.
Familial ovarian carcinoma.
AM J Med ; **81**: 1073-1076

Lynch, H. T., Bewtra, C., Wells, I. C., Schuelke, G. S., Lynch, J. F. 1987.
Hereditary ovarian cancer: clinical and biomarker studies. In:Lynch, H. T., Kullanders, editors. Cancer genetics in women.
Boca Raton, FL: CRC Press, PP 49-97

Margolis, B., Silvennoinen, O., Comoglio, F., Roonprapunt, C., Skolnik, E, Ullrich, A., and Schlesinger, J. 1992.
High-efficiency expression/ cloning of epidermal growth factor-receptor-binding proteins with Sre homology 2 domains.
Proc Natl Acad Sci USA ; **89** : 8894-8898

Marquardt, H., Rose, T. M., Webb, N. R. Hunkapiller, M.W., Hood, L.E., Tudor, G. J. 1984.
Rat transforming growth factor type structure and relation to epidermal growth factor.
Science ; **223**: 1079-1082

Martenz, N. D., Scaramuzzi, R. J., Vanlook, P., and Baird, D. T.1975.
Direct action of prostaglandins on the rat pituitary.
J Endocrinology ; **96**: 45-49

Marth, C., Lang, T., Koza, A., Mayer, I, and Daxenbichler, G.1990. Transforming growth factor-β and ovarian carcinoma cells; regulation of proliferation and surface antigen expression.
Cancer Lett ; **51**: 221-225

Massague, J., 1990
The transforming growth factor family
Ann Rev Cell Biol ; **6**: 597-641

May, J.V., Buck, P. A., Schomberg, D.W. 1987.
Epidermal growth factor enhances ^{125}I-follicle stimulating hormone binding by cultured porcine granulosa cells.
Endocrinology ; **120**: 2413-2420

McGuire, W. P., Rowinsky, E. K., Rosenshein, N. B. et al 1989.
Taxol, a unique anti-neoplastic agent with significant activity in advanced ovarian epithelial neoplasms.
Ann. Intern. Med ; **111**: 273-279

Mikiy., Swensen, J., Shattuck-Eidens, P. 1994.
A strong candidate for the breast and ovarian cancer suceptability gene BRCA1.
Science ; **266**: 66-71

Mondschein, J.S., Schomberg, D.W. 1980.
Growth factors modulate gonadotrophin receptor induction in granulosa cell cultures.
Science ; **211**: 1179-1180

Monroe, S. E., Menon, K. M. 1977.
Changes in reproductive hormone secretion during the climacteric and postmenopausal periods.
Clin. Obstet. Gynecol ; **20** : 113-122.

Mori, M., Harabuchi, I., Miyake, H. et al 1988.
Reproductive, genetic, and dietary risk factor for ovarian cancer.
Am J Epid ;**128** : 771-777

Mossmann, T. 1983.
Rapid calorimetric assay for cellular growth and survival, application to proliferation and cytotoxicity assays.
Journal of Immunological Methods ; **65** : 55-63

Muggia, F. M. 1989.
Overview of carboplatin: replacing, complementing, and extending the therapeutic horizons of cisplatin.
Semin Oncol ; **16** supply 5: 7-13

Naessen, T., Persson, I., Adam, H., Bergstrom, R., Bergkvisk, L. 1990.
Hormone replacement therapy and the risk for first hip fracture.
Ann Intern Med ; **113**: 95-103

Narod, S. A., Feunteun, J., Lynch, H. T., Watson, P., Conway, T., Lynch, J. Lenoir, G. M.1991.
Familial breast-ovarian cancer locus on chromosome 17q12-q23.
Lancet ; **338** : 82-83

Neijt, J. P., ten Bokkel Huinink, W.W., van der Burg, M. E. 1987.
Randomized trial comparing two combination chemotherapy regimens (CHAP-5 vcp) in advanced ovarian carcinoma.
J Clin Oncol ; 5: 1157-1168

Nicosia, S.V., Saunders, B. O., Acevedo-Duncan, M. E., Setrakian, S., Degregoria, R., 1991.
Biopathology of ovarian mesothelium. In: Familiar, G., Makabe, S., Motta, P. M., editors. Ultrastructure of the ovary.
Boston: Kluwers Academic Publishers ; 287-310

Nigro, J. M., Baker, S.J., Preisinger, A.C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Colins, F. S., Weston, A., Modali, R., Harris, C.C., Vogelstein, B. 1989.
Mutations in the P 53 gene occur in diverse human tumours types.
Nature ; **342**: 705-708

Oberkanins, C., Geurts-Moespot, A., Zeillinger, R., Kury, F., Leake, R. E., and Benraad, T. J. 1995.
The Performance of a Commercial Radioligand Binding Assay for the Epidermal Growth Factor Receptor is Comparable to the EORTC Standard Assay.
European Journal of Cancer ; **31A**, no 10 : 1710-1711

Osborne, C.K., Bolan, G., Monaco, M. E., and Lippman, M. E. 1976.
Hormone responsive human breast cancer in long term tissue culture: effect of insulin.
Proc Natl Acad Sci USA ; **73**: 4536-4540

Owens, O. J., Stewart,C., Brown, I. and Leake, R. E. 1991a.
Epidermal growth factor receptor in human ovarian cancer.
Br J Cancer ; **64** : 907-910

Owens, O. J., Stewart, C., Leake, R. E. 1991b.
Growth factors in ovarian cancer.
Br J cancer ; **64** : 1177-1181

Padhy, L.C., Shih, C., Cowing, D., Finkelstein, R., Weinberg, R. A. 1982.
Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas.
Cell ; **28**: 865-871

Piver, M. S, Baker, T. R., Piedmonte, M., Sandecki, A. M., 1991.
Epidemiology and etiology of ovarian cancer.
Semin Oncology ; **18**: 177-185

Poels, L. G., Ramaekers, F. F., Scheres, J.M., Thomas, C. M., Vooijs, P, G., et al 1989. Characterization of a hormone-producing ovarian carcinoma cell line:
Gynecol Oncol ; **32**: 203-314

Purchio, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L.E., Kovacina, K. S., Roth, R. A., Marquardt, H. 1988.
Identification of mannose 6-phosphate in two asparagine-linked sugar chains on recombinant transforming growth factors-β1 precursor.
J Biol Chem ; **263**: 14211-14215

Redford, H. M., Avenell, J. A., Panaretto, B. A. 1987.
Some effects of epidermal growth factor on reproductive function in merino sheep.
J Reprod Fertil ; **80**: 133-138

Reed, E., Kohn, K. W. 1990.
Cisplatin and platinum analogues in: chabner, B., A., Collins, J. M., editors cancer chemotherapy: principles and practice.
Philadelphia: J Blippincott ; 465-490

Reynolds, E. S. 1963.
The use of lead citrate at high pH as an electron opaque stain in electron microscopy.
J. Cell Biol. 17 : 208-212.

Richardson, G.S., Skully, R. E., and Nelson, J. H. 1985.
Common epithelial cancer of the ovary.
N Engl J Med ; **312**: 415-424

Roberts, A. B., Flanders, K. C., Kandaiah, P., Thompson, N. L., Van Obberghen-Shilling, E., Wakefield, L., Rossi, P., Dc combrugghe, B., Heine, U. and Sporn, M. B. 1988. Transforming growth factor-β: Biochemistry and roles in embryogenesis, tissue repair and remodelling and Carcinogenesis.
Rec Prog Horm Res ; **44**: 157-168

Roberts, A.B., Kim, S. J., Wakefield, L. M., Glick, A. B., Sporn, M. B. 1991.
TGF-β : altered transcriptional control and response patterns associated with Carcinogenesis.
American Association for Cancer Res ; **82**: 454-455

Rodriguez, G. C., Boente, M. P., Berchuk, A, Whitaker, R. S., O'Briant, K., Xu, F., Bast, R. C. 1993.
The effect of antibodies and immunotoxins reactive with the *HER-2/neu* proto-oncogene on growth of ovarian and breast cancer cell lines.
AM J Obstet Gynecol ; **43** : 55-60

Rodriguez, G. C., Berchuck, A., Whitaker, R. S., Schlossman, D., Clark-Pearson, D. L., Bast, R. C. 1991.
Epidermal growth factor receptor expression and response to epidermal growth factor.
AM J Obstet Gynecol ; **164** : 745-50

Ronin, C. 1989.
Gonadotrophin receptors.
Annales Endocrinology ; (Paris) **50**: 388-398

Ross, R. 1993.
The pathogenesis of atherosclerosis.
Nature ; **362**: 801-809

Runowicz, C. D. 1992.
Advances in the screening and treatment of ovarian cancer.
CA Cancer J Clin **42** (6) : 327-349

Russell, W. C., Newnam, C., and Williamson, D, H. 1975.
A sample cytochemical technique for demonstration of DNA in cells infected with mycoplasma and viruses.
Nature ; **353**: 461-462

Sabatini, D. D., Bensch, K. and Barrnett, R. J. 1963.
Cytochemistry and electron microscopy - the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation.
J. Cell Biol. **17**: 19-58

Sandgren, E. P., Luetteke, N.C., Palmiter, R.D., Brinster, R.L., and Lee, D.C. 1990.
Over expression of TGF-a in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast.
Cell ; **61**: 1121-1135

Sarosy, G., Kohn, E., Link, C. et al 1992.
Taxol dose intensification in patients with recurrent ovarian cancer.
Proc. Am. Soc. Clin. Oncol ; **11**: 716-717

Sato, T., Saito, H., Morita, R., Koi, S., Lee, J. H., Nakamura, Y. 1991.
Allele type of human ovarian cancer.
Cancer Res ; **51** : 5118-5122

Schechter, A. L., Hung, M. C., Vaidyanathan, L., Weinberg, R. A., Yang-Feng, T. L., Francke, U., Ulirich, A., Coussens, L. 1985.
The neu gene: an erb B-homologous gene distinct from and unlinked to the gene encoding the EGF-receptor.
Science ; **229**: 976-978

Schild kraut, J. M., Thompson, W. D. 1988.
Familial ovarian cancer.
A population-based case control study ;**128**: 456-466

Scurry, J. P., Hammand, K, A., Astley, S. B., Leake, R, E.,, Wells, M. 1994. Immunoreactivity of antibodies to epidermal growth factor, transforming growth factors alpha and beta and epidermal growth factor receptor in the premenopausal ovary.
Pathology ; **26** : 130-133

Show, G., Jorgenson, G.,Tweendale, R., Tennison, M.,Waters, M. J.1985.
Effect of epidermal growth factor on reproductive function of ewes.
J Endocrinol ; **107**: 429-436

Silverberg, E., Lubera, J. A. 1983.
A review of American cancer society estimates of cancer cases and deaths.
C. A ; **33** : 2-25.

Simard, J., Tonin, P., Durocher, F., et al 1994.
Common origins of BRCA1 mutaions in Canadian breast and ovarian cancer families.
Nat. Genetic ; **8**: 392-398

Simon P. Langdon, Sandra S. Lawrie, Frances G. Hay, Mary M. Hawkes, Amanda McDonald, Ian P. Hayward, Dick J. Schol, Jo Hilgers, Robert C. F. Leonard, and John F. Smyth. 1988.
Characterization and Properties of Nine Human Ovarian Adenocarcinoma Cell Lines.
Cancer Res ; **48**: 6166-6172

Singletary, S. E., Baker, F. L., Spitzer, G., Tucker, S. L., Tomasovic, B., Brock, W. A., Ajani, J. A., Kelly, A. M. 1987.
Biological effect of epidermal growth factor on the in vitro growth of human tumours.
Cancer Res ; **47**: 403-406

Skinner, M. K., Keski-oja, J., Osteen, K. G., and Moses, H. L., 1987. Ovarian thecal cells produce transforming growth factor-β which can regulate granulosa cells growth.
Endocrinology ; **121**: 786-792

Slamon, D, J., Clark, G. M., Wong, S.G., Levine, W.J., Ullrich, A., McGuire, W. L.1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2 /*neu* oncogenes.
Science ; **235**: 177-182

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J. Ullrich, A., Press, M. F.1989. Studies of HER-2 /*neu* proto-oncogenes in human breast and ovarian cancer *Science* ; **244** : 707-712

Smith, S. A., Eston, D. F., Evans, D. G. R., and Ponder, B. A. J. 1993. Allele losses in the region 17q12-q21 in familial breast and ovarian cancer non-randomly involve the wild type chromosome. *Nature Genet* ; **2** : 128-131

Sporn, M. B., Roberts, A. B., Wakefield, L. M., Assoian, R. K. 1986. Transforming growth factor-β Biological function and chemical structure. *Scince* ; **233** : 532-540

Stampfer, M. J., Colditz, G. A. 1991. Oestrogen replacement therapy and coronary heart disease; A quantitative assessment of the epidemiologic evidence. *Prev Med* ; **20**: 47-63

Stempak, J. G. and Ward, R. T. 1964. An improved staining method for electron microscopy. *J. Cell Biol.* **22** : 697-701

Susan, E., Hankinson, R. N., Graham, A. C., David, J. H., Terri, L. S., Bernard, R., Meir, T. S., 1992. A quantitative assessment of oral contraceptive use and risk of ovarian cancer. *Obstet Gynecol* ; **80**: 708-714

Takeshi Maruo, Cecilia, A., Ladines-Leave, Takashi Samoto, Hiroya Matsuo, Augusto, S. Manalo, Hiroshi Ito, and Matsuto Mochizuki. 1993.
Expression of Epidermal growth factor and its receptor in the human ovary during follicular growth and regression.
Endocrinology ; **132** : 924-931

Todaro, G, Fryling, C., Delarco, J. 1980.
Transforming growth factors produced by certain human tumour cells: polypeptides that interact with epidermal growth factor receptor.
Proc Natl Acad Sci USA ; 77: 5258-5262

Twardzik, D. R. 1985.
Differential expression of transforming growth factor-a during prenatal development of mouse.
Cancer Res ; 45: 5413-5416

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlesinger, J., Down ward, J., Mayes, E. L.V.,Whittle, N., Water field, M. D., and Seeburg, P. H.1984.
Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermal carcinoma cells.
Nature (Lond); **309**: 418-425

Ullrich, A., Schlesinger, J. 1990.
Signal transduction by receptors with tyrosine kinase activity.
Cell ; **61**: 203-212

Vessey, M. P., and Painter, R. 1995.
Endometrial and ovarian cancer and oral contraceptoves-findings in a large cohort study.
Br J Cancer ; **71** : 1340-1342

Vistica, D. T., Skehan, P., Scudiero, D., Monks, A., Pittman, A., and Boyd, M. R. 991.
Tetrazolium-based assays for cellular viability : a critical examination of selected parameters affecting formazan production.
Cancer Res ; **51** : 2515-2520

Wakefield, L. M., Smith, D. L., Flanders, K. C., Sporn, M. B. 1988. Latent transforming growth factor-β from human platelets.
J Biol Chem ; **263**:1646-1654

Whittemore, A. S. 1993.
Personal characteristics relating to risk of invasive epithelial ovarian cancer in older women in the United Stats.
Cancer ; **71** : 558-565

Whittemore, A. S., Harris, R., Itnyer, J., 1992.
Characteristics relating to ovarian cancer risk. Collaborative analysis of 12 US case-control studies. IV. The pathogenesis of epithelial ovarian cancer.
Am J Epidemiology ; **136** : 1212-1220

Whittemore, A. S., Wu, M. L., Paffenbarger, R. S., Sarles, D. L., Kampert, J. B., Grosser, S., Jung, D. L., Ballon, S., Hendrickson, M., Mohle-Boetani, J. 1989.
Epithelial ovarian cancer and the ability to conceive.
Cancer Res ; **49**: 4047-4052

Wiener, J. R., Hurteau, J. A., Whitaker, R. S., Kerns, B. J., Berchuck, A., and Bast, R. C. 1993.
Overexpression of the protein tyrosine phosphatase PTP 1B in human ovarian carcinomas.
Proc AM Assoc Cancer Res ; **34** : 527-534

Wilson, A. P. 1984.
Characterization of a cell line derived from the ascites of a patient with papillary serious cystadenocarcinoma of the ovary.
J Nat Cancer Inst ; **72** : 513 - 521

Wilson, A. P., Fox, H., Scott, I. V., Lee, H., Dent, M., Golding, P. R. 1991.
A comparison of the growth promoting properties of ascetic fluids. Cyst fluids and peritoneal fluids from patients with ovarian tumours.
Br J Cancer ; **63** :102-108

Wimalasena, J., Meehan, D., Cavall, C., 1982.
Human epithelial ovarian cancer cell steroid secretion and its control by gonadotropins.
Gynecol Oncol ; **41**: 56-6

Woodruff, J. D., Protos, P., Peterson, W. F. 1968.
Ovarian teratomas
Am J Obstet Gynecol ; **102**: 702-715

Yaish, P., Gazit, A., Gilon, C., and Levuter, C. itzki, A. 1988.
Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors.
Science (washington DC) ; **242**: 933-935